

**Oral Ulceration in Behçet's Disease:
An Investigation of
Neutrophil Elastase and Its Inhibitors**

Tanya Novak

**Thesis submitted to University of London for the Degree of
Doctor of Philosophy (PhD)**

December 2013

Bart's and the London Medical and Dental School
Centre for Clinical and Diagnostic Oral Sciences
The Blizard Institute for Cell and Molecular Science
Queen Mary, University of London

Supervised by
Dr. Eleni Hagi-Pavli
Prof. Farida Fortune

Acknowledgements

First and foremost I would like to thank Dr. Eleni Hagi-Pavli for all her support and guidance both personally and professionally. Your enthusiasm and hard work is inspiring – not just for me but all women in science.

I have immense appreciation for all my colleagues whose collective efforts at the busy clinics have never gone unnoticed. And thank you to Professor Farida Fortune for enabling the link between clinic and research and for her wise words and support.

I am indebted to the James Paget Studentship for without their financial support none of this would have been possible.

I would also like to personally thank Dr Sam Willcocks for believing in me and encouraging me throughout this challenging process. And to the entire Willcocks/Shearman family for embracing me as one of their own. And thank you to Lucy Sanders and her wonderful family for their rallied championing for me to get this thesis done!

And to my Mother, Pat, who has been an inspiration and shining light in my life. I thank her every chance I get for her support and unconditional love. You alone have provided me with the opportunities to be able to accomplish my goals throughout my life. I would not be where I am today without you.

And finally, I am very appreciative to the participants who took part in this study. From the beginning of this journey, my driving force has always been to help others. I hope the research I am fortunate enough to partake in is able to contribute towards good health!

Declaration

Required statement of originality for inclusion in research degree thesis

I, Tanya Novak, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged and my contribution indicated. Previously published material is also acknowledged.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

I accept that the College has the right to use plagiarism detection software to check the electronic version of the thesis.

I confirm that this thesis has not been previously submitted for the award of a degree by this or any other university.

The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without the prior written consent of the author.

Signature:

Date: 19/12/2013

Abstract

Behçet's disease (BD) is a vasculitis of unknown aetiology typified by recurrent oral and genital ulcers, skin and ocular lesions. Debilitating manifestations can also affect vascular, gastrointestinal and neurological systems. Previous BD investigations showed increased circulating neutrophils and neutrophil elastase (NE), a serine protease. NE can digest connective tissues compromising their integrity if not regulated. In this study, NE and its two main inhibitors, secretory leukocyte protease inhibitor (SLPI) and alpha1-antitrypsin (α 1AT), were investigated to determine if NE dysregulation is triggering oral mucosal damage. Findings were compared to healthy controls (HC) and recurrent aphthous stomatitis (RAS) patients, a disorder of episodic oral ulceration.

FlowCytoMixTM multiplex-assays compared saliva and serum inflammatory cytokines measurements where salivary levels reflected disease activity and correlated with published serum levels. Salivary NE, SLPI, and α 1AT were measured by ELISA. Patients with oral ulcers had increased NE. Unexpectedly, BDq (quiescent, without ulceration) had increased NE, but SLPI was significantly lower than RASq and HC. RASq NE levels were similar to HC. Overall, NE correlated with α 1AT levels, but showed an inverse relationship with SLPI. Quantitative PCR revealed significantly increased SLPI mRNA expression in both BDq and RASq buccal epithelium. High mRNA/low SLPI protein expression during ulceration could be explained by deficient translation, blocked ELISA antibody binding, or SLPI depletion. Despite high α 1AT, all study groups had enzymatically active salivary NE which was successfully inhibited by recombinant SLPI. Confocal microscopy revealed BD patients' blood neutrophils readily release neutrophil extracellular traps (NETs) *in vitro* compared to HCs. Antimicrobial NETs have mixed granule contents coating decondensed chromatin fibres and are associated with autoimmunity. During NET production, our novel observation that intracellular SLPI but not α 1AT co-localised with NE suggests a regulatory role.

This study supports the theory that a protease-antiprotease imbalance may play a role in BD oral and systemic pathology.

Abbreviations

a	Active oral ulceration (1 or more oral ulcers present)
Ab	Antibody
BD	Behçet's Disease
BDa	Behçet's Disease active (with oral ulcer(s))
BDq	Behçet's Disease quiet (no oral ulcer(s))
Bis-Tris	Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl
BLAST	Basic Local Alignment Search Tool
β-ME	βeta-mercaptoethanol
bp	Base Pairs
BSA	Bovine serum albumin
C	Celsius
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CF	Chemotactic factor
Cq	Quantification cycle
CrD	Crohn's Disease
DABCO	1,4-Diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ddH ₂ O	Double distilled H ₂ O
dH ₂ O	Distilled water
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECL	Electrochemiluminescence
ELISA	Enzyme linked immunosorbent assays
Fig	Figure
g	Grams
(x) g	Gravity (<i>see RCF</i>)
GCF	Gingival crevicular fluid
GFP	Green fluorescent protein
HC	Healthy control
HCl	Hydrochloric acid
H&E	Hematoxylin and eosin stain
HOCl	Hypochlorous acid

hr	Hour
IL	Interleukin
Ig	Immunoglobulin
IR	Infrared
IFN	Interferon
kDa	Kilodalton
mA	Milliamps
MES	2-(N-morpholino) ethane sulfonic acid
MGC	Magic Syndrome
MgCl ₂	Magnesium chloride
min	Minutes
mm	Millimeters
MPO	Myeloperoxidase
MW	Molecular weight
n	Sample size
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
ng	Nanogram
NTC	No template control
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
P.Ela.	Porcine pancreatic elastase
pg	Picogram
PMA	phorbol 12-myristate 13-acetate
PV	Pemphigus vulgaris
PVDF	Polyvinylidene difluoride
RAS	Recurrent aphthous stomatitis
RCF	Relative centrifuge force (or g)
REC	Research Ethics Committee
RT	Room Temperature
RT-qPCR	Reverse Transcriptase-Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RPM	Revolutions per minute
rSLPI	Recombinant secretory leukocyte protease inhibitor
rxn	Reaction
q	Quiet episode (no oral ulcer present)
QPCR	Quantitative polymerase chain reaction
Tris-HCl	Trizma base - Hydrochloric acid
SAL	Saliva
SDS	Sodium dodecyl sulfate
sec	Seconds
SEM	Standard error mean
SLPI	Secretory leukocyte protease inhibitor
TBS	Tris-HCl Buffer Solution
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
Tris	Trizma base
Tw-20	Tween [®] 20
U	Unit
μg	Microgram
μl	Microliters
μM	Micromolar
μm	Micrometers
U/rxn	Units per reaction
V	Volts
WB	Western blot

Table of Contents

Acknowledgements	2
Declaration.....	3
Abstract.....	4
Abbreviations	5
List of Tables	17
Chapter 1: Introduction and Aims	18
1. Introduction.....	19
1.1. Oral Ulceration with Non-Systemic Symptoms	19
1.1.1. Recurrent Aphthous Stomatitis	19
1.1.2. Neutrophils Implicated in RAS.....	20
1.1.3. RAS Treatment	20
1.2. Oral Ulceration and Systemic Disease: Behçet's Disease	21
1.2.1. Etiopathogenesis of BD	22
1.2.2. Histological Features of Oral Ulcers in BD and RAS	22
1.2.3. Current Treatments.....	23
1.3. The Mechanism of Oral Ulceration.....	24
1.3.1. The Oral Mucosa Epithelium	24
1.3.2. Saliva, Oral Homeostasis, and Ulceration	26
1.3.3. Saliva is Important for Oral Homeostasis	27
1.4. Hypothesis and Aims of the Project	28
Chapter 2: General Materials and Methods	29
2.1. Study Subjects, Specimen Collection, and Laboratory Processing.....	30
2.1.1. Subject Recruitment	30
2.1.2. Patient Information and Research Specimens	30
2.1.3. Collection and Processing Blood.....	31
2.1.4. Collection and Processing of Saliva Samples.....	31
2.1.5. Collection of Oral Buccal Mucosa Biopsies	32
2.1.5.1. Freezing Oral Biopsy Tissue	32
2.1.6. Collecting Oral Buccal Swabs.....	33
2.1.7. Isolating and Purifying RNA from OBS	34
2.1.7.1. Extraction and Purification of RNA	34
2.1.7.2. Initial Quality and Quantity of RNA	35
2.1.7.3. DNase Treatment and Reverse Transcription	35
2.1.7.4. Second Quality and Quantity Assessment of gDNA-free RNA	36
2.1.7.5. Reverse Transcription	36
2.1.8. Preparing Mowiol Mounting Media.....	37
Chapter 3: Inflammatory Cytokines in Saliva and Serum	38
3.1. Introduction.....	39
3.1.1. Cytokines and T-cells.....	39
3.1.2. Hypothesis and Aims of the Experiment	45
3.2. Methods.....	46
3.2.1. Cytokines and Chemokines in Saliva and Serum: The FlowCytomix™	46
3.2.1.2. The Sample Number for Saliva and Serum from Different Patient Groups.....	49
3.2.1.3. Statistical Analysis	49
3.3. Results	50
3.3.1. Pro-Inflammatory Cytokines in Saliva and Serum	50
3.4. Discussion.....	62
3.4.1. Saliva and Serum Inflammatory Markers	62
3.4.2. Conclusions:.....	67
Chapter 4: Neutrophil Elastase and Its Protease Inhibitors.....	68
4.1. Introduction.....	69

4.1.1. Neutrophil Elastase and its Main Protease Inhibitors: Secretory Leukocyte Protease Inhibitor and Alpha1-Antitrypsin	69
4.1.1.1. Neutrophil Elastase	69
4.1.1.2. Neutrophil Granules, NE and Exocytosis	69
4.1.1.3. Secretory Leukocyte Protease Inhibitor	73
4.1.1.4. Alpha1-Antitrypsin Inhibitor	73
4.1.1.5. Oral Mucosa Inflammation, Neutrophil Elastase and Regulation	74
4.1.1.6. NE, Disease, and Ulceration	77
4.1.1.7. Theories on the Mechanism by which SLPI and α 1AT Regulate NE: Forming Complexes	79
4.1.1.8. SLPI as a Potential Therapeutic Agent.....	87
4.1.1.9. α 1AT as a Potential Therapeutic Agent.....	87
4.1.1.10. SLPI Gene Expression	88
4.1.1.11. Inhibitor Inactivation and Other Regulatory Factors to Consider	89
4.1.2. Hypothesis.....	90
4.1.2.1. Objectives:	90
4.2. Materials and Methods	91
4.2.1. Western Blots for Detecting NE, SLPI, and α 1AT Proteins	91
4.2.1.1. Preparation of Samples and Positive Controls for Gel Electrophoresis	91
4.2.1.2. Transference of Proteins to Western Blot Membrane.....	91
4.2.1.3. Blocking Buffer	92
4.2.1.4. Washing Membranes	93
4.2.1.5. Western Blotting for SLPI	93
4.2.1.5.1. Primary SLPI Antibody	93
4.2.1.5.2. Secondary SLPI Antibody.....	93
4.2.1.6. Western Blotting for NE.....	93
4.2.1.6.1. Primary NE Antibody.....	93
4.2.1.7. Western Blotting for Beta-Actin.....	94
4.2.1.7.1. Human Beta-Actin Primary Antibody	94
4.2.1.7.2. Beta-Actin Secondary Antibody	94
4.2.1.8. Development and Detection Using the Dark Room ECL Method.....	94
4.2.2. Enzyme-Linked Immunosorbent Assays	95
4.2.2.1. Recovery Assays for SLPI, NE and α 1AT ELISA's	95
4.2.2.2. The Levels of NE, SLPI, and α 1AT in Saliva: ELISA Assays	96
4.2.2.2.1. Human SLPI Immunoassay	98
4.2.2.2.2. Human Elastase Kit	98
4.2.2.2.3. Human Alpha1-AntiTrypsin.....	99
4.2.2.2.4. Statistical Test for ELISA's, Enzyme Activity, and qPCR Assays.....	100
4.2.3. Protein Isolation from Oral Buccal Swabs	100
4.2.3.1. Initial Protein Isolation Protocol	100
4.2.3.1.1. Alternative Protein Isolation Protocol	100
4.2.4. Measuring Total Protein Concentration in Saliva and OBS Samples	101
4.2.5. Protease Enzyme Activity and Inhibition Assays	102
4.2.5.1. Principle of the EnzChek® Assay.....	102
4.2.5.2. Protease Activity Assays.....	102
4.2.5.2.1. Total Protease Activity in Saliva Samples	102
4.2.5.3. Inhibition Assays.....	103
4.2.5.3.1. The Inhibition of Purified NE by rSLPI (Proof of Principle).....	103
4.2.5.3.2. Testing the Inhibition of Endogenous NE in BDa Saliva Using Various Concentrations of Chloromethyl Ketone	104
4.2.5.3.3. Measuring Specific Neutrophil Elastase Activity in Saliva.....	104
4.2.6. QPCR for OBS SLPI and Reference Gene.....	105

4.2.6.1. Primer Design for Selected Reference Genes and Target mRNA	105
4.2.6.2. Initial Primer Testing	106
4.2.6.3. Producing cDNA for Use in Standard Curves: Purification of cDNA Standard.....	107
4.2.6.4. Optimisation of qPCR assay	108
4.2.7. Immunohistochemistry of Oral Biopsies	108
4.2.7.1. Sectioning Frozen Oral Biopsies.....	108
4.2.7.2. Giemsa or Hematoxylin and Eosin (H&E) Stain for Orientation	109
4.2.7.2.1. Giemsa Staining	109
4.2.7.2.2. H&E Staining	109
4.2.7.3. Fixing, Blocking, and Immunofluorescent Antibody Staining of Oral Biopsies.....	109
4.3. Results	111
4.3.1. Western blots: Identification of NE, SLPI, and α 1AT in Oral Specimens	111
4.3.1.1. Optimisation of Antibodies for Western Blot.....	111
4.3.1.1.1. Issues with Non-Specific Binding of Secondary Antibodies in Oral Specimens.....	111
4.3.1.1.2. Initial Monoclonal SLPI Primary Antibodies: No SLPI Detection in Saliva	114
4.3.1.3. The Detection of SLPI in Saliva, Saliva Cell Pellets, and Oral Buccal Swabs from HC and BD Patients with and without Oral Ulcers	116
4.3.1.4. Detection of NE in Oral Specimens	117
4.3.1.4.1. Detection of NE Using HRP-Labelled Primary Antibody.....	117
4.3.1.5. Detection of α 1AT in Saliva	120
4.3.2. Measurement of Salivary Levels of NE, SLPI, and Alpha-1 Antitrypsin using ELISA.....	121
4.3.2.1. The Levels of NE, SLPI, and α 1AT in Saliva from HC, BD and RAS Patients	121
4.3.2.2. NE, SLPI, and α 1AT Saliva Concentrations From BD Patients Taking Colchicine.....	130
4.3.3. NE and SLPI in Western Blots: Comparing ELISA Measurements	132
4.3.4. Total Protease and Specific NE Enzyme Activity in Saliva	134
4.3.4.1. Proof of Concept: Both Recombinant SLPI and a Chloromethyl Ketone Specific Inhibitor Can Prevent Purified NE from Hydrolysing Bovine Elastin Substrate	134
4.3.4.2. Total Protease Activity and Specific NE Activity in Saliva: Initial Tests....	137
4.3.4.3. Total Protease Activity in Saliva: Comparing Different Groups	140
4.3.4.4. Specific NE Activity in Saliva: Comparing Groups.....	141
4.3.5. Real Time Quantitative PCR	143
4.3.5.1. Validation and Controls	143
4.3.5.1.1. Bioanalyzer	143
4.3.5.1.2. Validated Reference Genes for Oral Epithelial Cells	144
4.3.5.1.3. Inhibition Assessment Assay	144
4.3.5.2. SLPI mRNA Expression Levels in OBS.....	146
4.3.6. Oral Biopsies Histology	148
4.3.6.1. NE and SLPI in Oral Ulcer Buccal Mucosa	148
4.4. Discussion.....	154
4.4.1. Salivary NE Levels in the Presence and Absence of Oral Ulcers	154
4.4.2. SLPI mRNA in Oral Buccal Epithelial Cells and Protein Level Discrepancy	154
4.4.3. The Inverse Relationship of NE and SLPI Protein.....	157
4.4.4. High Levels of Alpha-1 Anti-Trypsin in Saliva.....	159

4.4.5. Target Proteins Identified by Western Blots	162
4.4.6. Salivary Proteases and NE Activity.....	163
4.4.6. Distribution of NE in Ulcerated Oral Buccal Epithelium	165
4.4.6.1. Distribution of NE.....	165
4.4.6.2. SLPI in Ulcerated Mucosa	166
4.4.7. Conclusion	167
Chapter 5: NETosis in Behçet's Disease	169
5.1. Introduction.....	170
5.1.1. NETosis: The Discovery of an Alternative Type of Death in Neutrophils.....	170
5.1.2. The Role of DNA, Neutrophil Elastase and Myeloperoxidase in NETs.....	171
5.1.3. Endogenous Inhibitors of NETs	173
5.1.4. NETosis, or ETosis, in Other Granulocytes	174
5.1.5. The Pathology of Neutrophil Extracellular Traps.....	174
5.1.6. Aims.....	176
5.2. Materials and Methods.....	177
5.2.1. Optimisation Experiments	177
5.2.1.1. Initial Sytox® Green Experiments and Culturing Neutrophils on Poly-D- Lysine Coated Coverslips	177
5.2.2. Isolation of Neutrophils from Whole Blood for Investigation of NETs <i>In Vitro</i>	180
5.2.3. NET Formation	182
5.2.3.1. Immunocytochemical Qualitative Experiments: Antibody Staining and....	182
5.2.3.2. Quantification of Sytox Positive Neutrophils Preparing Neutrophils in Culture	186
5.2.4. Patients Included in the Study	189
5.3. Results	190
5.3.1. Investigating Neutrophil Extracellular Traps in Behçet's Disease: Sytox® Green Extracellular DNA Analysis	190
5.3.1.1. Immediate versus Next Day Imaging	190
5.3.1.2. Sytox® Green Analysis in Different Culture Conditions	194
5.3.1.2.1. Positive Control Experiments: Inducing Neutrophils Extracellular Traps using PMA Stimulant	194
5.3.1.2.2. Comparing Unstimulated Sytox+ Neutrophils from Different Patient.....	197
5.3.1.2.3. The Effect of NEi on Neutrophils	202
5.3.2. NETosis with and without PMA-Stimulation: Observation of Neutrophil Elastase <i>In Vitro</i> Using Immunocytochemistry	205
5.3.2.1. NE Inhibitors and NETosis	209
5.3.2.1.1. NE and its Synthetic Inhibitor	209
5.3.2.1.2. Endogenous Neutrophil-Derived SLPI During NETosis	211
5.3.2.1.2.1. NE and SLPI in Unstimulated Cultures	211
5.3.2.1.2.1.1. Unstimulated HC Blood Neutrophils <i>In Vitro</i>	211
5.3.2.1.2.1.2. Unstimulated Non-Relapsed BD Patients' Blood Neutrophils <i>In Vitro</i>	213
5.3.2.1.2.1.3. Unstimulated Relapsed BD Patients' Blood Neutrophils <i>In Vitro</i>	216
5.3.2.1.2.2. Unstimulated Neutrophil Cultures with NEi.....	218
5.3.2.1.2.2.3. Relapsed BD Patients' Blood Neutrophils <i>In Vitro</i> with NEi.....	221
5.3.2.1.2.3. PMA-Stimulated Neutrophil Cultures.....	223
5.3.2.1.2.3.1. PMA-Stimulated HC Blood Neutrophils <i>In Vitro</i>	223
5.3.2.1.2.3.2. PMA-Stimulated Non-Relapsed BD Patients' Blood Neutrophils <i>In</i> <i>Vitro</i>	225
5.3.2.1.2.3.3. PMA-Stimulated Relapsed BD Patients' Blood Neutrophils <i>In Vitro</i>	227
5.3.2.1.2.4. PMA-Stimulated Neutrophil Cultures with NEi.....	229

5.3.2.1.2.4.1. PMA-Stimulated HC Blood Neutrophils <i>In Vitro</i> with NEi	229
5.3.2.1.2.4.2. PMA-Stimulated Non-Relapsed BD Patients' Blood Neutrophils <i>In Vitro</i> with NEi.....	231
5.3.2.1.2.4.3. PMA-Stimulated Relapsed BD Patients' Blood Neutrophils <i>In Vitro</i> with NEi	233
5.3.2.1.2.5. Unidentified Cell Death	235
5.3.2.1.3. MPO and NETosis	236
5.3.2.1.4. The Role of Endogenous Alpha-1 Antitrypsin During NETosis.....	241
5.3.3. Summary of Key Results	244
5.4.1.3. Neutrophil Adherence to Non-Coated Glass Coverslips	247
5.4.1.4. The Different Morphology of NETs Created by Laboratory Handling	248
5.4.1.5. Immediate Imaging is Necessary for Accurate Sytox Quantification	248
5.4.1.6. Quantification of NETs.....	248
5.4.2. HC and Behçet's Disease Patients NETs	249
5.4.2.1. BD Patients' Neutrophils React Quickly to PMA Producing Enlarged Cells and Expansive NETs.....	252
5.4.2.2. NE Inhibitor Can Stop the Progression of NETosis <i>in Vitro</i>	253
5.4.2.3. The Role of Neutrophil-Derived α 1AT and SLPI	254
5.4.2.3.1. Intracellular Participation of SLPI but not α 1AT	254
5.4.2.3.2. Extracellular Co-Localisation of SLPI and α 1AT with NE	255
5.4.3. The Current Model of NETosis: NE, MPO, SLPI, and α 1AT	258
5.4.4. Eosinophils Did Not Undergo NETosis	259
5.4.5. Unidentified Death	259
5.4.6. Conclusions	260
Chapter 6: Validated Reference Genes for Ulcerated and Non-Ulcerated Oral Buccal Mucosa.....	261
6.1. Introduction.....	262
6.1.2. Reference Genes in RT-qPCR	262
6.1.4. Applying and Interpreting geNorm Analysis using qBasePLUS	265
6.1.5. Experiment Objectives	266
6.2. Materials and Methods.....	267
6.2.1. QPCR Roche Human Reference Gene Panel	267
6.3. Results	271
6.3.1. Most Stable Reference Genes in Oral Buccal Epithelial Cells.....	271
6.3.2. The RNA Integrity Assay	274
6.4. Discussion.....	277
6.4.1. Conclusions.....	280
Chapter 7: Final Discussion	281
7.1. Final Discussion.....	282
7.2. Final Conclusions	285
7.3. Future Work.....	286
Appendix 1.....	287
Appendix 2.....	296
References.....	298

List of Figures

Fig. 1.3.1.1: Layers of the Non-Keratinised Oral Mucosa.....	26
Figure 2.1.5.1.1. Orientation of biopsy for freezing.....	32
Figure 3.1.1.1. Cytokines mediate the immune response.....	38
Fig. 3.2.1.1. Demonstration of bead populations for different sizes and emissions on the flow cytometer.....	47
Fig. 3.3.1.1. IL-8, IL-1 β , and TNF- α concentration differences in saliva and serum from matched patients.....	51
Fig. 3.3.1.2. IL-2, IL-12p70, and IFN- γ concentration differences in saliva and serum from matched patients.	53
Fig. 3.3.1.3. TNF- β , IL-4, and IL-5 concentration differences in saliva and serum from matched patients.....	55
Fig. 3.3.1.4. IL-17A, IL-6, IL-10 concentration differences in saliva and serum from matched patients	57
Fig. 3.3.1.5. Correlation of IL-8 and IL-1 β measured in saliva from all patient groups.....	59
Fig. 3.3.1.6. Correlation of IL-12p70 and TNF- β measured in saliva from all patient groups.....	60
Fig. 3.3.1.7. Correlation of IL-5 and IL-10 measured in saliva from all patient groups...	61
Fig. 4.1.1.2.1. The Neutrophil and Primary, Azurophil Granules.....	72
Fig. 4.1.1.7.1. The Structure of SLPI Protein.....	76
Fig. 4.1.1.7.2. The Protein Structure of α 1AT and its Binding to NE.....	81
Figure 4.3.1.2.1. Detecting SLPI in Different Oral Specimens with the Polyclonal SLPI Primary Antibody.....	115
Figure 4.3.1.3.1. SLPI Detected in a Range of Oral Specimens from Three Patient Groups.....	116
Figure 4.3.1.4.1.1. A and B. NE Detected in Saliva, Blood, and Oral Buccal Swab Samples.....	118
Figure 4.3.1.5.1. α 1AT Detected in Saliva Samples.	120
Figure 4.3.2.1.1. Salivary NE levels amongst different patient group.....	122
Figure 4.3.2.1.2. Salivary SLPI levels amongst different patient groups.....	124
Figure 4.3.2.1.3. Salivary α 1AT levels amongst different patient groups	126
Figure 4.3.2.1.4. Comparison of SLPI, NE and Alpha1-Antitrypsin.....	127
Fig. 4.3.2.2.1. Comparing Saliva NE, α 1AT, and SLPI Concentrations from BD Patients Taking Colchicine.....	131

Figure 4.3.3.1. SLPI and NE in Saliva.....	133
Fig. 4.3.4.1.1. The Inhibitory Activity of rSLPI on the Enzyme Activity of NE: Testing Purified Proteins.....	136
Figure 4.3.4.2.1. Specific Inhibition of Endogenous NE in BDa Saliva: Testing Different Concentrations of Inhibitor (a Range of 25-150 μ M) with a BDa Saliva Sample	138
Figure 4.3.4.2.2. Specific Inhibition of Endogenous NE in BDa Saliva: Testing One Concentration of Inhibitor (25 μ M) with One BDa Saliva Sample at a Various Final Dilutions (Range 1:4 to 1:40).....	139
Figure 4.3.4.3.1. Comparing the Total Protease Activity in Saliva from Each Patient Group.....	140
Figure 4.3.4.4.1. Specific NE Enzymatic Activity in Saliva.....	142
Figure 4.3.5.1.1.1. A and B. A: Electropherogram of an RNA OBS Sample.....	143
Figure 4.3.5.1.3.1. Spud Inhibition Assay Results: cDNA Must Be Diluted Before Use in qPCR Assays.....	145
Figure 4.3.5.2.1. OBS SLPI mRNA Expression Levels in Different Patient Groups and Ulcer Sites.....	147
Fig. 4.3.6.1.1. Ora Ulcer Biopsy from Non-BD Patient Bx1.....	150
Fig. 4.3.6.1.2. Oral Ulcer Biopsy from BD Patient Bx2.....	151
Fig. 4.3.6.1.3. Oral Ulcer Biopsy from BD Patient.Bx3.....	152
Fig. 4.3.6.1.4. Oral Ulcer Biopsy from BD Patient Bx4.....	153
Figure 5.2.1.1.1. Initial Culturing Method: In-Well Imaging of Unstimulated Neutrophils from a BD Non-Relapsed Patient Cultured onto 0.001% Poly-D-Lysine Coated Glass Coverslips.....	179
Figure 5.2.1.1.2. Neutrophils from Relapsed BD Patient Cultured onto 0.001% Poly-D-Lysine Coated Glass Coverslips and Mounted.	179
Figure 5.2.2.1. A, B, C. A. The Layers of Blood after Histopaque Separation.....	181
Fig 5.2.3.2.1.1. Simultaneous Images in GFP and DAPI Channels.....	188
Fig. 5.3.1.1.1. A. and B. Representative Results of Increased Sytox+ Neutrophils During Next Day Imaging of the Same 1 hr PMA-Stimulated HC Neutrophil Culture.....	191
Fig. 5.3.1.1.2, A. and B. An Example of Increased Sytox+ Neutrophils During Next Day Imaging of the Same Unstimulated HC Neutrophil Culture	193
Fig 5.3.1.2.1.1. Inducing NETosis <i>In Vitro</i> using PMA: A Positive Control Experiment.....	195
Fig 5.3.1.2.1.2. The Difference in Time Points when Inducing NETosis <i>In Vitro</i> using PMA.....	196

Fig 5.3.1.2.2.1. The Mean Percentage of Sytox Positive Neutrophils in HC and BD patients.....	197
Fig 5.3.1.2.2.2. The Mean Percentage of Sytox Positive Neutrophils in Clinically Relapsed and Non-Relapsed BD Patients Compared to HC.....	198
Fig 5.3.1.2.2.3. The Mean Percentage of Sytox ⁺ Neutrophils: Comparing Patients On Azathioprine Medication.....	199
Fig 5.3.1.2.2.4. Comparing the Mean Percentage of Sytox ⁺ Neutrophils in Unstimulated Cultures at Two Different Time Points.....	201
Fig 5.3.1.2.3.1. Comparing the Mean Percentage of Sytox ⁺ Neutrophils in 1 hr and 3 hrs PMA-Stimulated Cultures with and without Neutrophil Elastase Inhibitor (NEi) in Different Patient Groups.....	203
Fig 5.3.1.2.3.2. Comparing the Mean Percentage of Sytox ⁺ Neutrophils in Unstimulated and Neutrophil Elastase Inhibitor (NEi) Treated Cultures in Different Patient Groups..	204
Fig 5.3.2.1. Positive Control: Neutrophils Undergoing NETosis in a PMA-Stimulated Culture.....	206
Fig 5.3.2.2. BD Patients' Neutrophils Undergoing NETosis in Unstimulated Culture.....	208
Fig 5.3.2.1.1. The Hypothesized Reaction to NE Inhibitor in Unstimulated and PMA-Stimulated NETosis.....	210
Fig 5.3.2.1.2.1.1. Unstimulated HC Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in Unstimulated Cultures.....	212
Fig 5.3.2.1.2.1.2. Non-Relapsed BD Patients' Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in an Unstimulated Culture.....	215
Fig 5.3.2.1.2.1.3. Relapsed BD Patients' Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in an Unstimulated Culture	217
Fig 5.3.2.1.2.2.1. Unstimulated HC Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in Unstimulated Cultures with NE Inhibitor.....	218
Fig 5.3.2.1.2.2.2. Non-Relapsed BD Patients' Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in an Unstimulated Culture with NE Inhibitor.....	220
Fig 5.3.2.1.2.2.3. Relapsed BD Patients' Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in an Unstimulated Culture with NE Inhibitor.....	222
Fig 5.3.2.1.2.3.1. HC Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in a PMA-Stimulated Culture.	224
Fig 5.3.2.1.2.3.2. Non-Relapsed BD Patients' Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in a PMA-Stimulated Culture.....	226

Fig 5.3.2.1.2.3.3. Relapsed BD Patients' Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in a PMA-Stimulated Culture.....	228
Fig 5.3.2.1.2.4.1. HC Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in a PMA-Stimulated Culture with NE Inhibitor	230
Fig 5.3.2.1.2.4.2. Non-Relapsed BD Patients' Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in a PMA-Stimulated Culture with NE Inhibitor.....	232
Fig 5.3.2.1.2.4.3. Relapsed BD Patients' Neutrophils <i>In Vitro</i> : Investigating the Occurrence of NETosis in a PMA-Stimulated Culture with NEi.....	234
Fig 5.3.2.1.2.5.1. HC and Non-Relapsed BD Patients' Neutrophils <i>In Vitro</i> : Undefined Cell Death Processes in Unstimulated Cultures.....	236
Fig 5.3.2.1.3.1. Positive Control: Neutrophils Undergoing NETosis in a PMA-Stimulated Culture.....	238
Fig 5.3.2.1.3.2. Unstimulated and PMA-Stimulated HC Neutrophils <i>In Vitro</i> : Observation of MPO.....	239
Fig 5.3.2.1.3.3. Unstimulated and PMA-Stimulated Non-Relapsed BD Neutrophils <i>In Vitro</i> : Observation of MPO.....	240
Fig 5.3.2.1.3.4. Unstimulated and PMA-Stimulated Relapsed BD Neutrophils <i>In Vitro</i> : Observation of MPO.....	241
Fig 5.3.2.1.4.1. Alpha-1Antitrypsin during NETosis <i>In Vitro</i>	243
Figure 5.4.3.1. Suggested in vitro model of unstimulated, sterile NETosis observed in our experimental system.....	258
Fig 6.2.1.3. The RealTime Ready Human Reference Gene 384-well plate.	270
Fig 6.3.1.1. Average Expression Stability of Reference Gene Targets.....	271
Fig 6.3.1.2. The Stability of GAPDH in BD and RAS patients with and without ulcers and HC.....	272
Fig. 6.3.1.3. Box Plot of Reference Gene Cq Results for all 7 Different Patient Group OBS Samples.	274
Fig 6.3.2.1. Demonstration of two different cDNA samples tested on the human reference gene panel for the continuous and equal reverse transcription of its RNA	275
Appendix 2.1. Antibody Staining Controls.....	297

List of Tables

Table 2.1.7.5.1. Mastermix Components for the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor.....	36
Table 3.2.1.2.1. Patient Demographics for Saliva and Serum Analysis.....	48
Table 3.3.1.1. The FlowCytomix™ Th1/Th2 Analyte Panel.....	49
Table 4.1.1.7.1. Summary of NE Details.....	85
Table 4.1.1.7.2. Summary of SLPI and α 1AT Details.....	86
Table 4.2.1.8.1. ECL Detection Solution and Incubation Time per Membrane.....	93
Table 4.2.2.2.1. Patient Saliva Sample Size (n) Tested on ELISA	97
Table 4.2.7.3.1. Antibody Details for Detecting NE and SLPI in Oral Mucosal Biopsies.	
Table 4.2.6.1.1. Primers Selected for OBS Sample Gene Analysis.....	106
Table 4.2.7.3.1. Antibody Details for Detecting NE and SLPI in Oral Mucosal Biopsies.....	110
Table 4.3.1.1.1.1. Secondary Antibodies Showing Specific and Non-Specific Binding in Human Saliva Samples.....	113
Table 4.3.1.4.1.1. HRP Conjugated NE Primary Antibody	117
Table 4.3.2.1. Patient Saliva Sample Number for Each ELISA.....	121
Table 4.3.2.2. Patient Groups Median Concentration Values of NE, SLPI, and α 1AT Salivary Protein Targets Measured by ELISA.....	127
Table 4.3.2.3. Value Range of the NE, SLPI, and α 1AT Salivary Protein Targets Measured by ELISA.....	128
Table 4.3.2.3.4. α 1AT in Serum.....	129
Table 4.3.6.1.1. Patient Descriptions and Biopsy Details.....	149
Table 5.2.3.1.1. Optimised Target Antibody Details, Blocking and Permeabilising Buffers, and Isotype Controls for PFA-Fixed Neutrophil NETs.....	184
Table 5.2.4.1. Patient Demographics for PFA-Fixed NETs Analysis.....	189
Table 5.3.3.1. Summary of Key Results *based on SLPI/NE staining data	244
Table 5.3.3.2. Differences and Similarities Collectively Observed When Comparing PMA-Stimulated and Non-Stimulated Neutrophils from All Patient Groups <i>In Vitro</i>	245
Table 6.2.1.1. Details of the OBS from Different Patient Groups, the Area Swabbed, and Sample Number (n) Tested on the Human Reference Gene Panel.. ..	267
Table 6.2.1.2. Preparing the cDNA Sample Mastermix for each OBS sample to be run on the Human Reference Gene Panel.	268
Table 6.3.1.4. The Mean Cq, Standard Deviation, and Standard Error Mean of the Six Most Stable Reference Genes in OBS.....	274
Table 6.3.2.1. The 5', middle, and 3' amplification results for all 35 cDNA samples included in the human reference gene panel	276

Chapter 1:

Introduction

and Aims

1. Introduction

1.1. Oral Ulceration with Non-Systemic Symptoms

1.1.1. Recurrent Aphthous Stomatitis

Oral ulceration is one of the most common oral disorders that can affect up to 25% of the global population (Scully and Porter, 2008). When oral ulceration occurs intermittently throughout a person's life without any other clinically related symptom, it is deemed recurrent aphthous stomatitis (RAS). The term aphthous (plural of aphtha) refers to the oral ulcer itself and stomatitis is the mucosal inflammation. The round or oval ulcer(s) are often painful and classified as minor (<10 mm diameter) in 80% of RAS cases which heal without any scarring. There can be single or multiple shallow ulcers with distinct, regular margins and have a grey, white, or yellow pseudomembrane with erythematous halos. Minor ulcers usually develop on non-keratinized mucosa such as the buccal and labial sites as well as the floor of the mouth and takes up to 2 weeks to resolve. About 10% of those with RAS suffer major ulcers (≥ 10 mm diameter) often lasting ~ 6 weeks typically on the throat, soft palate, and/or lips where scarring is more likely to occur. Herpetiform ulcers are rare (1-10% of RAS) and show up as widespread crops of 1-100 small 2-3 mm oral ulcers which fuse to create an irregular border. Similar to the minor form of ulceration, they can last about 7 – 10 days before healing usually without scarring (Natah et al., 2004, Scully and Porter, 2008).

Aphthous oral ulcers can also occur in a variety of immune disorders such as inflammatory bowel disease which includes Crohn's disease and ulcerative colitis (Fatahzadeh et al., 2009), neutropenia (Boxer and Dale, 2002), or due to chemotherapeutics (Scully and Felix, 2005). Therefore, a detailed medical history and clinical investigation is necessary especially if the patient has any systemic manifestations. Risk factors for developing an oral ulcer include poor oral hygiene, consuming spicy or acidic food, vitamin B12 deficiency, trauma, rubbing of prosthetics such as dentures, high stress levels, and the menstrual cycle (Talacko et al., 2010, Scully and Felix, 2005, Balan et al., 2012). This range of conditions shows that both local and systemic factors can influence the development of an ulcer, however the mechanism is still unknown.

1.1.2. Neutrophils Implicated in RAS

Primed neutrophil granulocytes of the innate immune system have been implicated in the recurrent manifestation of oral ulcers. RAS patients' neutrophils isolated from whole blood have been shown to produce significantly higher reactive oxygen species (ROS) levels than healthy controls (HCs). This was found in RAS patients with and without ulcers (Lewkowicz et al., 2003). Despite the histological detection of neutrophils at the site of an RAS ulcer, it has also been proposed that neutrophils are involved in the pathology of RAS due to diminished function. A study by Sistig, *et al*, demonstrated that circulating blood neutrophils from RAS patients with acute ulceration showed decreased migration *in vitro* compared to HCs (Sistig et al., 2001). However in another study by Dagalis, *et al*, there were no differences in RAS neutrophil chemotaxis (Dagalis et al., 1987). It has also been shown that RAS patients with and without ulcers had significantly decreased phagocytosis ability (Sistig et al., 2001). Interestingly, this diminished vital function was also true for saliva neutrophils from RAS patients with ulcers compared to HCs (Lukac et al., 2003) (Kumar et al., 2010) and individuals with the inflammatory condition oral lichen planus (Lukac et al., 2003). This suggests that in RAS patients, once neutrophils are recruited to the oral cavity, the cells' ability to ingest microbes is diminished. It is not clear whether salivary neutrophils from RAS patients favour extracellular anti-microbial action involving exocytosed granule proteases and ROS rather than intracellular microbicidity. There is limited literature of protease involvement in RAS ulcers, however, an increased plasma level of the potent neutrophil elastase (NE) proteolytic enzyme was measured in children with RAS (Polanska et al, 2006).

1.1.3. RAS Treatment

A clinical treatment regime is usually not necessary for minor aphtha, but may be sought for more severe major or herpetiform ulcers. Even though minor oral ulcers are fairly common, the frequency, size, and location of the ulcers can negatively affect one's quality of life (Mumcu et al., 2007). The main treatment goal is to relieve pain using topical anaesthetics therefore products containing lidocaine are frequently used (Altenburg et al., 2007). Local antiseptics and anti-inflammatories can be used as well. A controlled study showed that out of 54 different agents, Corsodyl® mouthwash containing chlorhexidine had the most beneficial effect of relieving oral ulcers (Edres et al., 1997). Antibiotics such as tetracycline may also be prescribed. In more severe cases, the topical use of steroids may be prescribed but this may increase the risk of *Candida albicans* infection (Vincent and Lilly, 1992). Systemic therapy is an option for severe RAS

sufferers but is usually reserved for individuals who also have a systemic inflammatory involvement (see Section **1.2.3**).

1.2. Oral Ulceration and Systemic Disease: Behçet's Disease

The main investigative patient group in this study have Behçet's disease (BD) in which oral ulcers are a primary feature. BD is a rare, multi-systemic disorder which is thought to be partly autoimmune and autoinflammatory in its nature causing lesions on mucosal surfaces and the skin as well as affecting the ocular, cardiovascular, neurological, gastrointestinal systems and/or joints (Mendes et al., 2009). Skin problems, such as erythema nodosum and folliculitis, may also feature. BD patients do not necessarily develop all of these systemic symptoms which is partly why BD is so difficult to diagnose. Vascular involvement that can lead to thrombosis and aneurysms are the main causes of death among BD patients (Kural-Seyahi et al., 2003). The development of uveitis can lead to blindness. Neurological maladies of the central nervous system may develop such as meningo-encephalitis and neuro-psychiatric symptoms, although involvement of the peripheral nerves and muscles is rare (Al-Otaibi et al., 2005). The multiple manifestations suffered by individuals with BD can be debilitating.

Oral ulcers are believed to develop as a consequence of the systemic disease. There are many aspects of BD that as yet need to be explained such as the causative factors and why the illness, in most cases, initially manifests in the oral cavity in the form of recurrent ulcers in 86.5-100% of the cases (Al-Otaibi et al., 2005, Mumcu et al., 2009). The clinical diagnosis for BD is based upon the collective criteria published in 1990 by the International Study Group for Behçet's Disease in which a patient would present with recurrent aphthous oral ulcers at least three times per year (the types described in section **1.1.1**) plus at least two additional symptoms characteristic of the disease, for instance, eye lesions, skin lesions, genital ulcers, and/or a positive cutaneous pathergy test (1990). BD patients have a higher incidence of major and herpetiform ulcers than RAS (Main and Chamberlain, 1992, Oh et al., 2009). A laboratory-based diagnostic test has not yet been discovered for BD hence only after extensive investigation, often involving various professional disciplines, and excluding differential diagnoses, can a clinician conclude that a person has BD.

1.2.1. Etiopathogenesis of BD

Several microbiological entities such as *Helicobacter pylori*, *Streptococcus sanguinis*, and Herpes simplex virus I have been implicated in the pathogenesis of BD but never decisively proven (Avci et al., 1999, Studd et al., 1991, Yokota et al., 1992). BD patients have also been described as having hypersensitivity to the presence of microbes (Kurauchi et al., 2005).

Hyperfunctional neutrophils are characteristic of BD leading to an overactive neutrophil response (Matsumura and Mizushima, 1975) (Sakane et al., 1999). Various neutrophil chemotaxis and priming factors are found to be up-regulated in BD such as interleukin (IL)-8, tumour necrosis factor alpha (TNF- α), (Mege et al., 1993) and IL-1 β (Yosipovitch et al., 1995). Furthermore, significantly increased levels of NE in plasma has been detected in both quiescent and active symptomatic episodes of BD (Deger et al., 1995).

Other reported findings associated with the immunopathogenesis of the disease are inflammatory reactions due to human heat shock protein and the tendency towards a T helper (Th)1-type cytokine profile in active BD (Kurokawa et al., 2004). The strongest evidence of any genetic risk factor is the existence of human leukocyte antigen (HLA)-B51 allele on the major histocompatibility complex (MHC) locus, but this genotype is not detected in all BD cases (Kurokawa et al., 2004). BD has been deliberated to be a hereditary disorder due to its high incidence rate in specific geographical regions and the demonstration of affected generations within a family (Fresko et al., 1998). However, a genetic cause has not been ascertained.

1.2.2. Histological Features of Oral Ulcers in BD and RAS

BD and RAS patients share nearly indistinguishable histological features of oral ulceration (Lehner, 1978). Their oral ulcer biopsies are mainly described as non-specific ulceration showing a large infiltration of neutrophils, some macrophages, and mast cells (Direskeneli, 2006) (Kose et al., 2008) (Natah et al., 2004). Gamma-delta ($\gamma\delta$)-T-cells may also be present which are rarely seen in normal oral mucosa (Natah et al., 2000, Pepin et al., 1993). Both BD and RAS ulcers are also positive for CD4 and CD8 T cells, as well as the Th1-type cytokines IL-12, interferon-gamma (IFN- γ), and TNF- α . However, IL-4, a Th2 cytokine, has only been detected in BD ulcers (Dalghous et al., 2006). Recently, BD oral ulcers were also found to express the pro-apoptotic protein, Bcl-2, at high levels which could encourage inflammatory cell survival but has also been

implicated in the autoimmune disease systemic lupus erythematosus (Kose et al., 2008) (Tischner et al., 2010).

1.2.3. Current Treatments

BD cannot be “cured” therefore the treatment revolves around controlling the symptoms. Oral ulcers are treated similarly to RAS patients which includes topical immunosuppressants, ointments and mouthwashes containing anaesthetic, anti-microbial, and anti-inflammatory agents. A common recommendation is Betnesol[®] mouthwash containing the moderately potent corticosteroid betamethasone phosphate to control inflammation. Steroid-based, antiseptic topical creams are also used for genital ulcers. In addition, depending on the organs and areas of the body affected, medication is usually necessary if symptoms are more difficult to alleviate. Colchicine was first suggested by Matsumura and Mizushima in 1975 when increased neutrophil chemotaxis was first recognised in BD (Matsumura and Mizushima, 1975) and has been effective in the treatment of oral, genital, and ocular ulceration (Sander and Randle, 1986). Colchicine acts to reduce neutrophil migration (Roubille et al., 2013). It can be found binding to tubulin in blood leukocytes and inhibits microtubule polymerization. Downstream, this alteration, reduces the E-selectin ligand distribution on endothelial cells and the expression of L-selectin on neutrophils – both of which are necessary as part of the neutrophil transendothelial migration from the blood stream to tissues (Witko-Sarsat et al., 2000, Cronstein et al., 1995). Colchicine is also commonly used for rheumatic inflammation due to gout (Roubille et al., 2013) and so is prescribed to BD patients with arthritic manifestations as well as mucocutaneous disease (Al-Otaibi et al., 2005).

Thalidomide can also reduce neutrophil chemotaxis, TNF- α production from a variety of cells, and the development of new vessels associated with vascular inflammation (Altenburg et al., 2007). It has been shown to be effective in reducing severe recurrent oral ulceration (Revuz et al., 1990) and orogenital and follicular skin lesions in BD (Hamuryudan et al., 1998). However, a major risk factor for female patients who may become or are currently pregnant is malformation of the foetus (Altenburg et al., 2007).

A monoclonal antibody against TNF- α (anti-TNF- α) is a biological agent also known as infliximab and is fairly new in its use for BD (Marshall, 2004). It has shown improvement of refractory uveoretinitis (Okada et al., 2012), uveitis and extraocular manifestations (Accorinti et al., 2007), orogenital ulceration, and gastrointestinal symptoms.

Azathioprine, an immunosuppressive drug, has been shown to reduce systemic inflammation in all aspects of the disease including uveitis, oral and genital ulcers, and arthritis (Yazici et al., 1990, Keogan, 2009). Azathioprine blocks CD28 signalling, a co-stimulatory molecule on CD4 T cells, resulting in the cell's apoptosis thus down regulating the adaptive immune response (Maltzman and Koretzky, 2003). Mycophenolate mofetil is also an immunosuppressive sometimes used in place of azathioprine for mucocutaneous (Adler et al., 2001) and neurological BD symptoms (Shugaiv et al., 2011).

Non-steroidal anti-inflammatory drugs (NSAIDs), are useful in cases involving arthritis, but have been shown to cause oral ulcers as a side effect (Boulinguez et al., 2000). Similarly, the disease-modifying drug methotrexate has also shown a high incidence of oral mucosal damage (Kalantzis et al., 2005). Systemic corticosteroids, such as prednisolone, can be used for an acute, symptomatic flair involving inflammation and to regulate pain, but are not recommended for long term use as the side effects can be detrimental. A regime can include several of these medications at one time. All medications have a degree of toxicity manifesting as adverse side effects and therefore are not without long term risks.

1.3. The Mechanism of Oral Ulceration

1.3.1. The Oral Mucosa Epithelium

Human oral keratinocyte cells make up the majority of the outer-most layer of the oral mucosa epithelium, or stratified squamous epithelium, the mucosal surface barrier (**Fig. 1.3.1.1**). Oral keratinocytes may or may not be keratinized (expressing the structural protein keratin) depending on the region of the mouth (Moharamzadeh et al., 2007); for instance, the buccal mucosa is made up of non-keratinized cells whereas the gingival epithelium is partially keratinised. Keratinocytes provide structure and barrier protection from the external environment. The oral buccal epithelium has an intermediate thickness of 40-50 cell layers and, because it lacks keratin, allows easy diffusion of serum proteins into the oral cavity and likewise is more permeable to extracellular components (Shojaei, 1998). In response to external stimuli such as microbes, oral keratinocytes have been shown to produce IL-1 α , IL-6, transforming growth factor (TGF)- β , TNF- α , basic fibroblast growth factors, (Formanek et al., 1999) as well as granulocyte macrophage colony stimulating factor (GM-CSF) (Yamamoto et al., 1994). They also produce IL-8, a potent inflammatory chemotatic protein which can recruit neutrophils as part of the innate immune response and induce the release of NE.

Fibroblasts, the most common cells of the connective tissue, are congregated in the lamina propria just below the epithelial top layer (**Fig 1.3.1.1**). They produce collagen and extracellular matrix which supports the overall tissue structure. Fibroblasts also play an important role in wound healing and tissue remodelling. This may be assisted by the collective fibroblast and keratinocyte production of secretory leukocyte protease inhibitor (SLPI), a protective anti-microbial and serine protease regulating protein, which has also been shown to help in scarless oral wound healing (Sumi et al., 2000). Fibroblasts also have a strong influence on keratinocyte adhesion, keratin expression, and epithelial phenotype depending on the original location and nature of the fibroblast (Okazaki et al., 2003). They can be prompted to produce IL-8, IL-1 α , IL-6, and IFN- γ . Fibroblasts synthesize keratinocyte growth factor, fibroblast growth factors (Knerer et al., 1999), hepatocyte growth factor, and have receptors for epidermal growth factor (EGF) (Wang et al., 1990). These proteins assist in keratinocyte differentiation and epithelium regeneration (Gron et al., 2002). It has been shown that salivary EGF is reduced in RAS and BD patients even when there is no ulcer present (Adisen et al., 2008). This could lead to mucosal damage due to a lack of natural reparative processes.

Up to 10% of the oral mucosa epithelium is made up of other cell types including melanocytes, Merkel cells, and Langerhans cells and deeper into the submucosa there is a small number of macrophages and inflammatory cells such as lymphocytes as well as plasma cells and mast cells (Moharamzadeh et al., 2007). Langerhan cells, a type of intraepithelial dendritic cell, although fewer in number than keratinocytes, act as important antigen presenting cells (APC) along with macrophages (Shojaei, 1998). They both have MHC class II molecules, as do neutrophils, which can prime naïve T helper cells (Barrett et al., 1996) to produce IL-2 which induces T-cell proliferation and cytokines such as IFN- γ , GM-CSF, IL-4, and TNF- β (Walker, 2004).

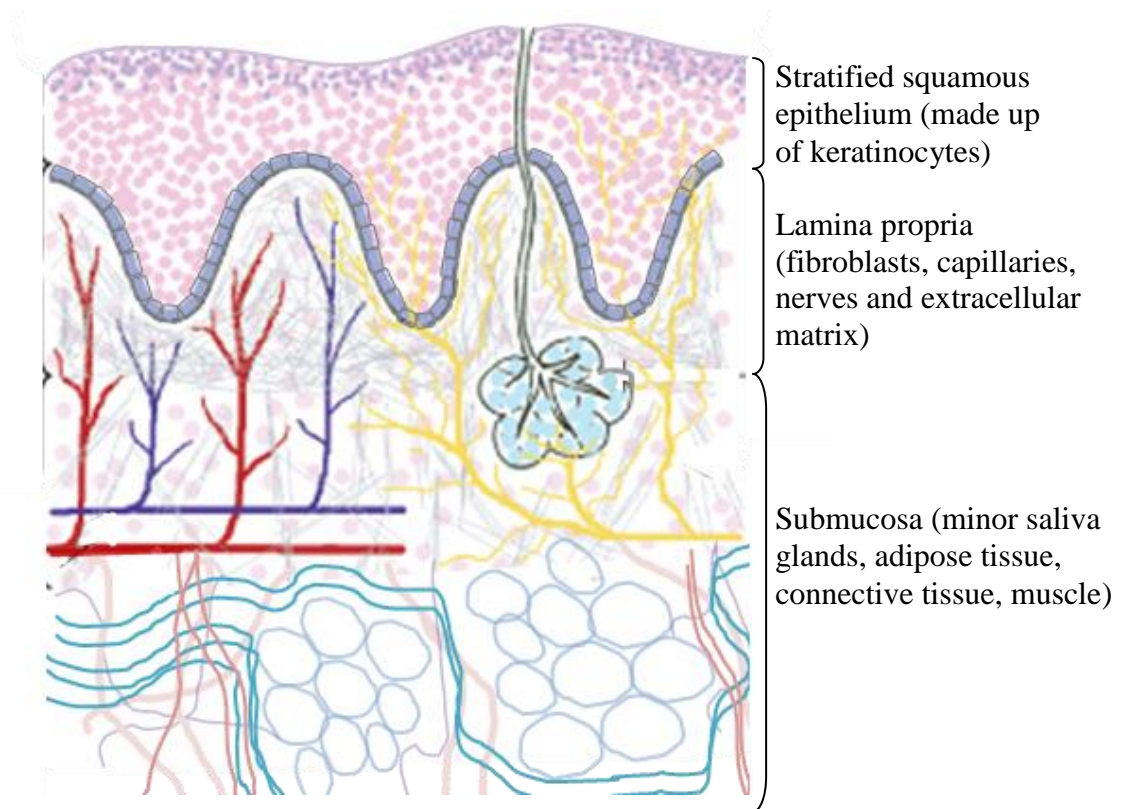


Fig. 1.3.1.1: Layers of the Non-Keratinised Oral Mucosa

The apical layer of the oral mucosa is the stratified squamous epithelium. Non-keratinized epithelium lines the buccal mucosa, inner lips, ventral surface of the tongue, floor of the mouth and soft palate. Desquamation readily occurs at the surface while the basal cells proliferate and move upward. Below this, the lamina propria incorporates the connective tissue components made up of the extracellular matrix (ECM) including elastin and collagen as well as capillaries, fibroblasts, and inflammatory cells (macrophages, mast cells, and neutrophils). The submucosa is also made up of connective tissue as well as adipose tissue and anchors minor saliva glands. The figure is based on an illustration from Ireland (Ireland, 2006) which has been updated and reconfigured for this thesis.

1.3.2. Saliva, Oral Homeostasis, and Ulceration

A critically important component that allows cells of the oral mucosa to function normally is saliva. It is a complex biological fluid that provides digestive enzymes to breakdown food, interacts with commensal or foreign bacteria, viruses, and fungi, provides a medium on which immune cells and complexes travel, and maintains good oral health. It is also a lubricant between soft tissues and hard enamel of the teeth which reduces irritation (Schipper et al., 2007). Individuals who have abnormal production of saliva, such as dry mouth, suffer from increased tooth decay, gingivitis, and gum inflammation, and reoccurring oral ulcers as seen in Sjögren's syndrome (Talacko et al., 2010).

Three major glands contribute to the salivary content: the parotid, submandibular, and sublingual glands (Miller et al., 2010). In whole saliva, minor salivary glands (on the tongue, palate, lips, and cheeks), as well as gingival crevicular fluid (GCF), and passive diffusion of serum-derived molecules from capillaries adjacent to the glands contribute to the fluid components (Miller et al., 2010, Schipper et al., 2007). The main constituent of saliva is water (99.5%) followed by glycoproteins, enzymes (such as NE), peptides, immunoglobulins such as secretory IgA (Walker, 2004) and inorganic electrolytes (Miller et al., 2010). These many salivary constituents and the sloughed off cells of the lining layers of the mucosa such as epithelial cells on the tongue, buccal, labial, hard and soft palate are constantly being incorporated into saliva. Needless to say, the mouth is a very active environment. There is also plenty of biochemical activity from the surrounding cells and bacteria making saliva an ideal specimen for cell and protein research. Saliva is also easy to collect, non-invasive, pain-free, cost effective, and is also safer to collect than blood as there is a low infectivity risk to the patient and health professional (Lee and Wong, 2009).

1.3.3. Saliva is Important for Oral Homeostasis

Throughout an ulcer's existence in the mouth, from its development to healing stage, it is bathed in saliva that is laden with proteins such as enzymes and growth factors. However, there is an uncertainty whether oral mucosa is behaving "normally" in individuals with RAS and systemic disorders like BD. It is of clinical interest to investigate what is occurring in the saliva and mucosa in the time between non-ulcerated tissue and an ulcer's development. It is currently unknown what biochemical injuries the mucosa is enduring in these disorders and if it involves systemic elements from the blood vessels just beyond the mucosa lining, the local cells making up the mucosal epithelium or from the salivary gland secretions.

1.4. Hypothesis and Aims of the Project

The project presupposes on the basis of the evidence presented above that NE dysregulation is causing oral mucosal damage in BD and RAS patients and thereby triggering recurrent oral ulceration. Hence, the aims of this study are to:

1. Determine whether levels of inflammatory markers measured in serum are also reflected in saliva and whether these levels correlate to disease activity. Saliva may be a reliable, non-invasive clinical specimen allowing BD patient to be monitored.
2. Investigate whether the enzymatic activity and levels of NE are increased in BD and RAS during oral ulceration.
3. Measure the endogenous protease inhibitors, alpha1-antitrypsin (α 1AT) and secretory leukocyte protease inhibitor (SLPI) in BD and RAS during active and quiescent oral ulcer episodes in order to determine whether levels are adequate to control NE.
4. Determine whether BD neutrophils undergo cell death via the novel mechanism of NETosis in which neutrophil extracellular traps (NETs) are released contributing NE to the local environment.

Chapter 2:

General

Materials and

Methods

2.1. Study Subjects, Specimen Collection, and Laboratory Processing

2.1.1. Subject Recruitment

Adults aged 18 years and above being investigated for or confirmed to have BD or RAS were recruited to the study with informed consent (REC number P/03/122) (See **Appendix 1**). Individuals with RAS were allocated as a disease control group. Samples were collected during multidisciplinary BD clinics which occurred twice a month: once at St Thomas Hospital and the other on-site at the Oral Medicine Clinic at the Dental Hospital, Royal London in Whitechapel. For the last year of the study, the local BD Clinic occurred fortnightly and the St Thomas site was no longer attended for sample collections. Patients frequently had repeat visits which enabled comparative experiments of the patient's disease activity at different time points. A separate clinic at the Whitechapel site was allocated for RAS patients and HCs.

2.1.2. Patient Information and Research Specimens

Specimens collected for analysis consisted of whole unstimulated saliva, oral buccal mucosa swabs, and oral tissue biopsies. Blood was collected into 5 millilitres (ml) or 10 ml heparin EDTA anti-coagulator vials for the isolation of plasma, PBMC, and neutrophils while coagulating vials were used for serum. Each participant's personal information was recorded such as their name, date of birth, sex, ethnicity, the presenting clinical activity of their systemic disease (if applicable) as well as the absence or presence and severity of an oral ulcer (minor, major, or herpetiform) using a clinical activity log sheet that was completed by the examining clinician (See **Appendix 1**: Behçet's Disease Clinical Activity Information Forms). For our specific study criteria, if one or more oral ulcers were present during specimen donation, then the patient was deemed orally active (a). If there was no ulcer, they were recorded as having a quiet (q) episode. Patients were also assessed on their systemic activity during clinic and deemed systemic active, or having relapsed, if they had at least three symptoms characteristic of the disease, for instance, oral ulcers, joint inflammation, eye or skin lesions or genital ulcers. This clinical assessment was based on the Criteria for Diagnosis of Behçet's Disease by the International Study Group for Behçet's Disease . If there were no systemic symptoms or complaints, they were deemed as systemically quiet. Each participant was designated a unique and confidential identification number and samples were labeled with this code and additional abbreviation of SAL, PLA, SER for saliva, plasma and serum,

respectively, along with the clinic date before storage at -80°C. Recorded patient information and completed questionnaires were kept secure in a locked metal cabinet in a university building which requires ID card access. A central database was created for patient and specimen information and a copy supplied on an encrypted password-only accessible USB.

2.1.3. Collection and Processing Blood

Blood was collected for NHS clinical processing and research at the same time. Venepuncture was performed and blood collected directly into Vacutainers® (Becton, Dickinson Co. UK). Blood was collected into one 5 ml tube which coagulated it in order to derive fibrinogen-free serum as well as two 10 ml EDTA non-coagulating purple tubes for plasma, PBMC, and neutrophil isolation (See NETs protocol **Section 5.2.2.**).

For serum collection, the BD Vacutainers® were centrifuged to separate the whole blood from the serum at 3300 x g for 6 minutes (min) at room temperature (RT). Serum was aliquoted into 300 µl into a 2 ml cryovial. Freezer-proof labels were placed on each vial with the patient's study code number, patient group (HC, BD, or RAS), specimen type and the date of sample collection and clinic. Samples were stored at -80°C until use.

2.1.4. Collection and Processing of Saliva Samples

Patients and HC volunteers rinsed their mouth with 5 ml of water prior to collection of unstimulated whole saliva. Patients were asked to expectorate over a maximum period of 5 minutes into 20 ml sterile universal tubes which were immediately placed on ice during clinic. The volume of saliva collected from each patient varied from 1-2 ml. Samples were transferred to the laboratory, centrifuged at 4°C for 15 minutes at 3500 x g in order to remove cellular debris. The saliva supernatant was then aliquoted, 200 µl per vial, avoiding the cellular pellet. Unless otherwise stated, when discussing a saliva sample in subsequent experiments, this refers to the cell-free saliva supernatant portion that was aliquoted. The saliva samples were placed immediately on dry ice to freeze as was the left over cell pellet. Samples were stored at -80°C until use.

2.1.5. Collection of Oral Buccal Mucosa Biopsies

Patients attending BD clinic who presented with oral ulceration and which warranted further investigation as deemed by the oral medicine consultant were referred to a specialised biopsy clinic. Informed patient consent was obtained. Clinical patient details were recorded (**Appendix 1: Oral Biopsy Clinic Patient Details Form**). After the tissue was excised, the biopsy was placed briefly on a sterile petri dish and portioned in half - each half keeping a portion of un-ulcerated epithelium. One half was sent for histological analysis by a pathologist (Prof Kim Piper) and the other half was prepared immediately as described below.

2.1.5.1. Freezing Oral Biopsy Tissue

In order to prepare the biopsy for freezing in liquid nitrogen ($N_2(l)$), the biopsy needed to be orientated so that when being cut, it would give a cross section of epithelium, lamina propria and submucosa layers. A square glass coverslip was wedged vertically into a small round cork where it had been slightly perforated with a scalpel (**Fig 2.1.5.1.1**). A few drops of optimum cutting temperature (OCT) (Tissue-Tek®, Sakura® Finetek, VWR, UK) was placed onto the cork where the biopsy was to be embedded and the tissue was orientated so that the epithelium was adjacent to the coverslip. Finally, a few more drops of OCT were added onto the top of the biopsy and firmly holding the cork with long tweezers, the entire cork and tissue were submerged into $N_2(l)$ for 10 sec to freeze the tissue. When complete, the coverslip was removed and the cork snapped along the perforated area. The biopsy fixed to the cork was then placed into a cryovial labelled with the patient ID number and date and transported to the laboratory either in $N_2(l)$ or on dry ice. The tissue was stored in the $-80^{\circ}C$ freezer.

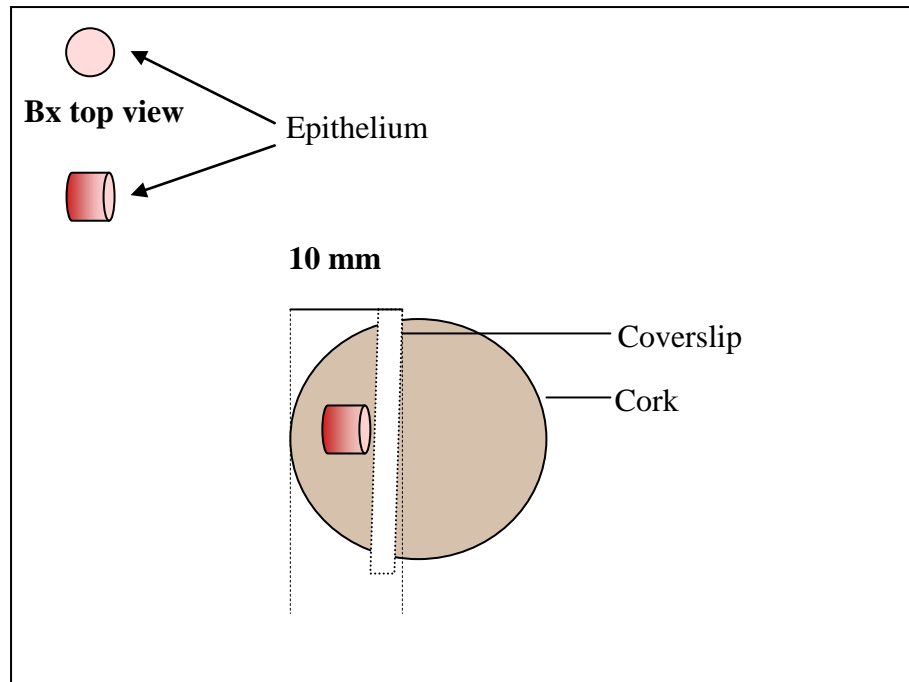


Figure 2.1.5.1.1. Orientation of biopsy for freezing. A small cork base was perforated with a scalpel and a glass coverslip slotted in. The biopsy was adjusted so that the epithelium was adjacent to the erected glass coverslip. The entire conformation was immersed into liquid nitrogen using long forceps.

2.1.6. Collecting Oral Buccal Swabs

Oral mucosa buccal swabs (OBS) were obtained from the inner cheek of patients. The 20 mm sterile cytology brush (Deltalab, Spain) is able to collect the upper layers of the mucosal epithelium without damaging tissue. Each brush was swiped 10 times over an area of the mucosa. The cellular material on the brush was immersed into a 1.5 ml microcentrifuge tube with 400 µl of RLT Buffer (RNeasy Micro Kit, Qiagen, UK) which contains the chaotropic salt guanidine thiocyanate to disrupt cell membranes and preserve nucleic acid. The brush was vigorously agitated in the buffer to disperse the cells then discarded and using a new sterile brush a second swab was taken from the same area and collected into the same lysis buffer as the first swab. Approximately 50 µl of the lysis buffer is lost during the process of immersing the brushes to remove cells leaving 350 µl of sample for extraction. The procedure was then repeated on the opposite inner cheek with a new 400 µl aliquot of RLT Buffer. If a patient had an oral ulcer and approved, the swab was gently swept over the ulcer and recorded as a direct ulcerated (U) mucosal sample. Samples from non-ulcerated areas were recorded as (NU). Specimens were immediately frozen on dry ice whilst at clinic in order to preserve the RNA content. Back in the lab, samples were stored at -80°C until RNA and protein extraction could be completed.

2.1.7. Isolating and Purifying RNA from OBS

2.1.7.1. Extraction and Purification of RNA

The initial, critical step involved when defrosting the OBS cells in lysis buffer on ice, was the addition of 3.5 μ l (1% final concentration) of beta-mercaptoethanol (β -ME) to the 350 μ l sample and vortexing to homogenize. The β -ME acts as an RNase inhibitor and therefore protects the RNA from degradation during subsequent procedures, especially those performed at room temperature. The RNeasy Micro Kit (Qiagen, UK) was utilized for the extractions as described by the supplier's protocol. After the lysate was homogenized, 20 ng of carrier RNA supplied by the kit was added to the sample. Then, one volume of 70% ethanol (made up in RNase-free water, Life Technologies, UK) was added to the homogenised lysate and mixed. The sample solution was then pipetted into a MinElute spin column with 2 ml collection tube and centrifuged at 16,000 x g for 1 min at room temperature which bound the RNA (and residual DNA) in the sample to the silica column. The flow through containing DNA and protein was saved for downstream experiments.

The nucleic acids bound to the column were washed with 350 μ l of Buffer RW1 centrifuging at the same time and speed as the previous step. An on-column DNase digest was then performed. For each sample, 10 μ l of reconstituted DNase I (2.73 U/ μ l) and 70 μ l of Buffer RDD were combined in a separate tube and then a total of 80 μ l was added to the top centre of each column. The column was left for 15 min at room temperature and then washed with 350 μ l of Buffer RW1 after which the column was centrifuged at 12,000 x g for 1 min. The flow through was discarded and the column was placed into a new 2 ml collection tube and 500 μ l of Buffer RPE (which was pre-diluted with ethanol) was added to the top of the column. The column was again centrifuged at 12,000 x g for 1 min and the flow through discarded. Another wash with 500 μ l of 80% ethanol was added to the top of the column and centrifuged for 2 min at 12,000 x g. The collection tube was replaced again and the column centrifuged at 16,000 x g for 5 min. Afterwards, the collection tube and contents were discarded. Lastly, the spin column was placed into a new 1.5 ml microcentrifuge tube and 14 μ l of RNase-free water was added to the centre of the column, left for 1 min, and centrifuged at 16,000 x g for 1 min to elute the RNA.

2.1.7.2. Initial Quality and Quantity of RNA

The eluted RNA was immediately placed on ice. To assess the RNA quantity and quality a Nanodrop™ 1000 spectrophotometer (Thermo Scientific, UK) was used to measure the Absorbance (A) at 260nm and 280nm where a 260/280 ratio between 1.7 - 2.0 indicated a good purity for an RNA sample. The Nanodrop™ can accurately measure 2 – 3700 ng/μl of double stranded (ds)DNA and 2 – 3000 ng/μl of RNA as determined by the manufacturers.

2.1.7.3. DNase Treatment and Reverse Transcription

A second DNase enzyme digestion of genomic (g)DNA was performed on the RNA eluate before undergoing reverse transcription (RT). Degrading all residual gDNA in the sample was critically important in order to accurately quantify the targets. Also, the design of the primer sequences for all 19 reference targets were unknown (Roche proprietary information). They may or may not be intron-spanning regions of the gene and could potentially amplify a portion of the gDNA sequence.

In order to degrade all gDNA, the TURBO™ DNA-free kit (Ambion, UK) was utilised. Each DNase reaction was composed of 5 μl of 10X TURBO buffer, 1 μl TURBO DNase enzyme (2 Units/μl), 11 μl of extracted OBS sample and RNase-free water to make a final 50 μl final reaction volume. The sample did not exceed 2 μg of nucleic acid (since 2 Units of TURBO DNase is able to remove a maximum of 2 μg gDNA in a 50 μl reaction). The mixture was briefly vortexed, pulse centrifuged, and placed on a PCR heat block for 30 min at 37°C. Afterwards, samples were taken off the heat block and 5 μl of DNase inactivation reagent from the kit was added to each sample. This was incubated for 5 min at room temperature with occasional vortexing to resuspend the sediment. The samples were then centrifuged at 10,000 x g for 1.5 min. The clear supernatant fluid (purified RNA) was collected by pipette carefully avoiding the white pelleted material and transferred to a new clean tube and kept on ice. Approximately 48 μl of isolated RNA was recovered.

2.1.7.4. Second Quality and Quantity Assessment of gDNA-free RNA

In order to further assess the quality and quantity of the gDNA-free RNA, 1 µl of up to 11 RNA samples at a time were tested on the Agilent 2100 Bioanalyser using an Agilent RNA 6000 Pico Chip (Agilent Technologies) for measuring Eukaryote Total RNA. The procedure was followed as described in the manufacturer's manual. Once the RNA samples were loaded, the chip was read within 5 min. Based on the results of the electropherograms showing the 18S and 28S rRNA peaks, the quality of the samples were assessed. If the RNA sample had a poor RNA integrity number (RIN) and showed major degradation, then the sample would be excluded from any further downstream analysis.

2.1.7.5. Reverse Transcription

Based on the concentration and volume of RNA available, RT was performed on ≤ 2 µg per 20 µl reactions with the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Life Technologies, UK). In most instances, the reaction volume was doubled to 40 µl in order to incorporate 20 µl of sample (since most RNA samples had low protein concentrations). Reagents from the kit were defrosted and kept on ice. A mastermix was created by mixing the reagents listed in **Table 2.1.7.5.1**.

Reaction Component	Starting Concentration (provided by the kit)	Final Concentration in Mastermix
Reaction Buffer	10X	1X
deoxynucleotide triphosphates mix (dNTP)	25X (100 mM)	1X (4 mM; 1000 µM each)
RT Random Primers	10X	1X
MultiScribe RT Enzyme	50 U/µl	2.5 U/µl
RNase Inhibitor	20 U/µl	1 U/µl

Table 2.1.7.5.1. Mastermix Components for the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor.

The final volume was adjusted to 40 µl by adding DNase/RNase-free water. The mixture was vortexed and 20 µl was distributed to new 0.2 ml eppendorfs containing 20 µl of RNA sample. (Note that an aliquot of 2 µl of each RNA sample was kept at -80°C for assessment of gDNA contamination on the reference panel described in Chapter 6). Each RT reaction tube was then placed into a Veriti Thermo Cycler PCR machine (Thermo Scientific, UK) with the following incubation times and temperatures: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min to deactivate the RT enzyme, and cooled to 4°C

until retrieved and placed on ice. The new complementary (c)DNA was aliquoted, labelled, and placed into a -20°C freezer.

2.1.8. Preparing Mowiol Mounting Media

Mowiol mounting media was prepared several days before use. Mowiol[®]4-88 was generously supplied to us by Dr Ann Wheeler. All other reagents were supplied by Sigma, UK. In order to prevent the fluorophores fading on the mounted specimen, 0.4 % of 1,4-Diazabicyclo[2.2.2]octane (DAPCO) was also included in the preparation. To make 25 ml of Mowiol, 6 g of glycerol, 6 ml of sterile dH₂O, 12 ml of 0.2 M Tris (pH 8.5), and 0.1 g of DAPCO were combined with 2.4 g of Mowiol[®] 4-88 in a glass flask and mixed with a magnetic stirrer for between 5-7 hours and then allowed to sit for 2 hrs (the Mowiol does not dissolve completely). It was then incubated at 50°C for 10 min and centrifuged at 5000 x g for 15 min in 2 ml eppendorf tubes or top speed in a large bench top centrifuge. The mixture's supernatant was then aliquoted and frozen at -20°C until use. The Mowiol solution was allowed to equilibrate to room temperature before use otherwise air bubbles formed under the coverslip.

Chapter 3:

Inflammatory Cytokines

in Saliva and Serum

3.1. Introduction

3.1.1. Cytokines and T-cells

Lymphocytes derived from the bone marrow which develop in the thymus, otherwise known as T-cells, make up the fundamental cells of the cell-mediated immune response. A portion of these migrate through the blood to secondary lymph organs such as the lymph nodes, spleen, tonsils, and epithelium-associated tissue such as Peyer's patches in the gastrointestinal tract, and areas of the lungs and skin. It is usually here or during recirculation that the T-cells are exposed to foreign proteins or polysaccharides by antigen-presenting cells. This process activates T-cells and a variety of cell to cell signalling proteins or peptides, called cytokines, modulate their recruitment to sites of inflammation (Alberts, 1994).

Two main types of T-cells exist: cytotoxic T-cells (with CD8 co-receptor) and helper T-cells (with a CD4 co-receptor). Cytotoxic T-cells recognise infected cells which present foreign antigen on a specialised protein called major histocompatibility factor (MHC) Class I and subsequently directly kill the cell. However, helper T-cells do not kill the antigen presenting cell they recognise with MHC Class II molecules outright. Instead, they contribute to the immune response by stimulating other white blood cells. This prompt is coordinated by a variety of local mediators: cytokines and chemokines.

Prior to their own production and secretion of cytokines, naïve T helper cells can undergo differentiation into several different subtypes depending on protein signals from the environment (**Fig 3.1.1.1.**). For instance, if interferon (IFN)- γ and/or interleukin (IL)-12 are increased in the surrounding area, they will promote the development of type 1 helper T cells, or Th1. These in turn continue to produce additional IL-12 and IFN- γ as well as IL-2, TNF- α , and TNF- β (Palmer, Weaver, 2010). One of the main duties of Th1 cells is to help activate macrophages, cytotoxic T-cells, and Natural Killer (NK) cells. If the external protein signals are predominately IL-4, this will persuade the development of type 2 helper T cells, or Th2. This type of T-cell will continue to produce IL-4 as well as IL-5, IL-6, and IL-10 mostly interacting with eosinophils, some T-cells, mast cells, and antibody producing B-cells leading towards humoral type immunity.

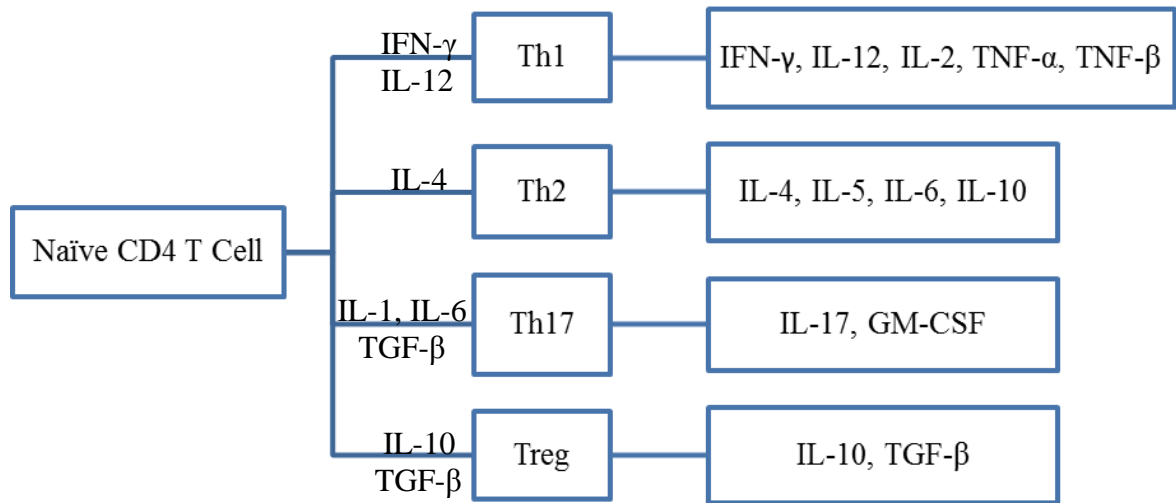


Figure 3.1.1.1. Cytokines mediate the immune response. Depending on which cytokine is dominant in the environment drives the development towards Th1, Th2, Th17, or Treg responses. In turn, the cells involved in these pathways release specific cytokines to help regulate, overcome, and resolve a pathogen or host's immune imbalance. Figure is adapted from (Mesquita Jr et al., 2009). It is important to note that these cytokines are mainly but not exclusively produced by CD4 T-cells.

IL-2 is a Th1-type cytokine that is produced mainly by helper T-cells. IL-2 can stimulate self-proliferation of helper and cytotoxic T-cells and B cells and is therefore at times considered a type of growth factor (Alberts, 1994). Increased levels of IL-2 in the plasma as well as a significant increase of its receptor has been detected in lymphocytes from RASa patients (Sun et al., 2000).

IL-12p70 is a Th1 pro-inflammatory cytokine made up of a 40 kDa and 35 kDa subunits. It is produced by macrophages, B-cells, and dendritic cells in response to foreign antigens such as LPS, intracellular microbes, or activated T-cells. IL-12 has also been known as the natural killer cell stimulatory factor and the cytotoxic lymphocyte maturation factor mediating cell killing via NK cells and T cells. It can induce IFN- γ and IL-2 production and helper T-cell proliferation leading towards a Th1 immune response. An increase of IL-12 in BD serum has been correlated with BD activity (Turan et al., 1997) and disease progression (Frassanito et al., 1999).

IFN- γ was originally recognised as the main constituent of the Th1 response. It is produced mostly by the same cells that produce IL-2 but can also be generated by cells of both the innate and acquired immune systems, namely NK cells and T-cells. IFN- γ targets B cells, macrophages, and endothelial cells activating the expression of Class II MHC molecules on the surface of these antigen presenting cells (APC) (Alberts, 1994). Although BD is often recognised as a Th1 dominant response (Frassanito et al., 1999), there are studies that have conflicted with the classic generation of increased IFN- γ . For instance, IFN- γ was measured in serum of BD active patients and was found to be lower than BD quiet, RAS, and HC (Aridogan et al., 2003).

Tumour necrosis factor-alpha (TNF- α) is a rapidly released cytokine that can act as an alarm when encountering foreign or stress stimuli (Tracey et al., 1987). It is mainly produced by monocytes and macrophages but can also be sourced from T-cells, neutrophils, mast cells, and endothelial cells (Feldmann and Maini, 2001). As the name implies, it can induce the death of tumour cells but also has an important role of initiating an immune response (via chemotaxis) and can be pyrogenic (causing fever). Acute up-regulation of the immune response is usually beneficial, however chronic exposure to TNF- α can impair T-cell activation as seen in *in vitro* studies (Cope et al., 1994).

An *in vitro* study has shown that TNF- α was increased in unstimulated peripheral blood monocytes from active RAS patients than in HC (Taylor et al., 1992). Oral lesion and non-lesional sites from RAS patients have also been shown to have increased levels of TNF- α mRNA as well as IL-2 and IFN- γ mRNA compared to HC (Buno et al., 1998). Thalidomide (which reduces TNF- α activity) and pentoxifylline (which acts to inhibit TNF- α production) have been effective in RAS ulcer treatment (Revuz et al., 1990, Zabel et al., 1993). TNF- α has been one of the most investigated cytokines in BD (Zhou et al., 2012). Studies continue to test this cytokine and its levels in different BD manifestations, for example, a recent study found that serum from BD patients with active uveitis have been shown to have significantly higher levels of TNF- α than those without any eye involvement for 3 months (Ozdamar et al., 2009). Anti-TNF- α therapy (such as Infliximab) has also been helpful in controlling active episodes of uveitis in BD (Hatemi et al., 2009) and oral and genital ulceration (Robertson and Hickling, 2001). All of these findings imply that TNF- α has an important role in RAS and BD pathologies.

Tumor necrosis factor-beta (TNF- β) is also known as lymphotoxin and is a potent inflammatory cytokine similar to TNF- α . It is produced by activated T and B lymphocytes and can be toxic to tumor cells. In cytotoxicity tests, TNF- β was more cytotoxic than TNF- α in a murine fibroblast cell line but less toxic against capillary endothelial cells (Kircheis et al., 1992). In BD, it has been found to be secreted in high levels by $\gamma\delta$ T cells when compared to HC (Yamashita et al., 1997) but is infrequently tested in the majority of BD and RAS investigations.

IL-4 and IL-5 are Th2-type cytokines produced by helper T-cells. They both stimulate B cells to proliferate and mature. IL-5 also promotes maturation of eosinophils. IL-4 is considered a critical element for driving the development of Th2 immune response (Choi and Reiser, 1998). Neither cytokine has been well characterised in BD (Zhou et al., 2012) nor have they been investigated extensively in RAS. There is some data available from a study conducted by Aridogan, *et al*, (Aridogan et al., 2003) in which the IL-4 (and IL-10) serum levels were increased in active BD.

IL-6 is produced by T-cells, B cells, and macrophages and can stimulate the proliferation of B cells and immunoglobulins as well as differentiate T lymphocytes (Yamakawa et al., 1996, Hirano et al., 1986, Alberts, 1994). A naïve T-cell can be stimulated by IL-6 (and transforming growth factor beta (TGF- β) to become a Th17-type cell which in turn releases IL-17A (Shimizu et al., 2012). An increased level of IL-17A has been detected in BD serum (Chi et al., 2008) and several other autoimmune diseases such as rheumatoid arthritis (Benedetti and Miossec, 2013). It has also been found infiltrating the salivary glands in Sjögren's syndrome (Alunno et al., 2013) and in the plasma from those with systemic lupus erythematosus (Ambrosi et al., 2012). In addition to CD4⁺ Th17 cells, IL-17 (with ligand A) is produced by $\gamma\delta$ T cells, NK cells (Raifer et al., 2012), and CD3⁺ (CD4⁻/CD8⁻) cells (Alunno et al., 2013) in response to extracellular pathogens and can induce the production of IL-8 thus recruiting neutrophils and contributing to inflammation. However when the release of IL-17A is exacerbated, this can also lead to the destructive tissue pathologies of inflammatory and autoimmune diseases (Mellett et al., 2012). Recently, BD has been investigated for production levels of IL-17. In 2008, Chi, *et al*, reported that stimulated PBMC's from BD patients with uveitis had increased levels of IL-17 but this could not be detected in the patient's serum (Chi et al., 2008).

IL-10 is also a key immunoregulatory cytokine produced by almost all leukocytes which include the innate immune cells (monocytes, macrophages, dendritic cells, mast cells, NK cells, eosinophils, and neutrophils) and adaptive immune cells (T-helper cells Th1 and Th2, CD8+ T-cells, B cells, and $\gamma\delta$ T cells) and Th17 type cells. While IL-10 can help to regulate the Th1/Th2 balance, it can also promote development of the Th2 type response by inhibiting IFN- γ being made in T-cells (Romagnani, 1995). It further promotes antibody production in B cells by increasing Bcl-2 expression which decreases B cell apoptosis (Levy and Brouet, 1994). IL-10 has been found in high levels in active BD patient serum (Aridogan et al., 2003) but has not necessarily been significantly increased in highly active BD compared to inactive or mild disease (Turan et al., 1997).

IL-8 is a potent chemokine whose presence in low levels can elicit a strong immune response to a variety of stimuli especially for the recruitment of neutrophils and activation of leukocytes in response to bacterial antigens. It is produced by a multitude of cells including neutrophils, monocytes, macrophages, endothelial and epithelial cells. Neutrophils and lymphocytes express the IL-8 receptor (Murphy and Tiffany, 1991) as do monocytes, NK cells (Morohashi et al., 1995), human umbilical vein endothelial cells (HUVEC) (Murdoch et al., 1999), human lung microvascular endothelial cells (HMVECs), and dermal human microvascular endothelial cell lines (HMECs) (Schraufstatter et al., 2001). IL-8 works to enhance the adherence of circulating leukocytes to endothelial cells during inflammation (Sahin et al., 1996).

IL-8 has been one of the main chemokines studied in BD. The serum concentration of IL-8 has been detected in significantly high amounts in BD patients compared to HC but not consistently between active and inactive episodes of BD (Sahin et al., 1996). Durmazlar, *et al*, sought to differentiate BD with vascular problems and correlation with serum IL-8. They found that active BD with vascular involvement had a 4-5 fold increase of IL-8 over those without any vascular symptoms (such as thrombosis or superficial thrombophlebitis). IL-8 in inactive BD patients with a history of vasculitis was still 2-fold higher than inactive BD with no vascular association (Durmazlar et al., 2009). It has also been found that an increase of IL-8 mRNA was directly associated with BD serum-treated macrophages from HC *in vitro* (Alpsoy et al., 2003). Mantas, *et al*, suggested that although there was a diverse source of IL-8 from BD cells, lymphocytes were considered to be the major contributors (Mantas et al., 2000).

IL-1 β is recognised as a “master cytokine” in the process of inflammation for being the main mediator coordinating an attack on invading microbes (Dinarello, 1996, Cauci et al., 2003) or in response to injury. It is produced mainly by blood monocytes, but can also derive from macrophages, dendritic cells, and neutrophils increasing fever and hypotension. IL-1 β can induce many chemokines, especially IL-8, and mediates systemic inflammation (Dinarello, 1996). It has been observed that an increase in IL-1 β has a simultaneous increase in IL-8 as well. IL-1 β has been investigated in BD, but not to the extent of IL-8 or TNF- α . A significant rise of the protein in the serum has been detected from BD active and inactive patients compared to HC (Duzgun et al., 2005).

Although the main investigation of this experiment was to identify pro-inflammatory cytokines associated with oral and systemic disorders, some of the cytokines on the panel have dual roles acting as pro- and anti-inflammatory proteins to down-regulate the immune systems reaction. These cytokines include IL-4, IL-6, and IL-10 which also make up the Th2-type response and have been shown to usually be lower in concentration than Th1 type cytokines in the BD immune response. IL-4 and IL-10 can inhibit macrophage function. Some Th1 and Th2 cytokines can be cross-regulatory, such that Th1 type cytokines like IL-12 and IFN- γ can inhibit Th2 activation whereas IL-4, a Th2 type cytokine, can inhibit IFN- γ production (Elenkov, 2002) (Lucey et al., 1996).

Persons with BD have had their blood plasma and serum repeatedly investigated for markers of inflammation in order to gain understanding of the mechanism of their immune response. Research conducted in many different countries including but not limited to Turkey (Akdeniz et al., 2004), Saudi Arabia (Sheth et al., 1995), Japan (Yamakawa et al., 1996), and Europe (Zouboulis et al., 2000) have measured proteins associated with inflammation in their patient cohort, however, the majority have relied on the same laboratory method of a sandwich ELISA. While this is still considered a reliable technique, protein levels in serum samples are often too low to be measured accurately, if at all (Yamakawa et al., 1996, Zouboulis et al., 2000).

Cytokines in saliva have rarely been investigated in BD or RAS. As a test specimen, unstimulated whole saliva has many benefits. It is readily available, non-invasive, and hosts a mass of salivary gland and cell-derived proteins in the fluid. Granted, the mouth is not a sterile environment. Saliva envelopes the natural flora of the mouth and will contain microbial contaminants which may influence the local cellular immune response. Another

potential bias when using saliva as a test specimen for inflammation may be an individual's overall oral health and hygiene. However, incorporating diverse group of healthy controls for cytokine comparisons should help to resolve any potential bias and result in a valid interpretation.

3.1.2. Hypothesis and Aims of the Experiment

It is believed that saliva will reflect the systemic inflammation in BD patients serum but not RAS patients. In order to test this theory, this experiment aims to compare saliva and serum and their respective inflammatory profiles in HC, BD, and RAS to:

1. Differentiate pro-inflammatory proteins between patients with and without oral ulcers
2. Investigate if BD systemic inflammation can be detected in saliva while no oral ulcer is present
3. To identify specific cytokines or chemokines that may influence the onset of an ulcer
4. To investigate if there is a correlation between inflammatory markers in serum and saliva
5. To validate the use of FlowCytomix™ array with saliva

3.2. Methods

3.2.1. Cytokines and Chemokines in Saliva and Serum: The FlowCytomix™ Multiplex Array

The FlowCytomix™ Multiplex array (Bender MedSystems, UK) is a bead based assay system in which microbeads of different sizes are coated with specific fluorescent antibodies to detect different cytokines in a sample. This allows multiple target detection using a low volume of sample and is compatible with various flow cytometers. An investigation of the inflammatory cytokines and chemokines (IL-8, IL-1 β , IL-2, IL-12p70, IFN- γ , IL-10, IL-6, IL-4, IL-5, TNF- α , TNF- β , and IL-17A) were measured in 25 μ l of the cell-free portion of neat unstimulated saliva and matched serum from BD, RAS, and HC participants. The protocol was followed as described in the manufacturer's manual for the human Th1/Th2 kit (Bender MedSystems, eBioscience, Ltd, UK).

To reconstitute the standards, a small volume of dH₂O was added to the 12 individual analyte vials as indicated on the label. 10 μ l of each standard was then added to a clean 1.5 ml eppendorf (Standard 1) resulting in a volume of 120 μ l to which 80 μ l of 1X array buffer was added for a final volume of 200 μ l, this is an initial 1:20 dilution of each analyte. 100 μ l of array buffer was added to a further 6 eppendorf tubes (Standards 2-7) and then 50 μ l of Standard 1 was added to Standard 2 and vortexed to mix. This 1:3 dilution series was completed for the rest of the standards and repeated in duplicate. 25 μ l of the duplicate Standards 1-7 were then transferred to labelled FACS tubes. An additional third tube for Standard 1 was also included and used for setting up the flow cytometer.

Next, the Bead Mix was prepared. For each sample, standard, and blank, 25 μ l of the bead mix was required. Depending on the final volume of the mixture, a final dilution of 1:20 of each bead solution was added to the mix and vortexed. The final volume was made up with reagent buffer and then the mix was centrifuged for 5 min at 3000 x g in order to wash the beads. Supernatant was discarded and replaced with reagent dilution buffer to achieve the same final volume then vortexed. A solution of Biotin-Conjugate was also prepared by adding 50 μ l per reaction (including standards, blanks, and samples) into a new tube for a final dilution of 1:20 in reagent buffer therefore for 12 analytes, 300 μ l of Biotin-Conjugate was added to 2400 μ l of reagent dilution buffer).

To new pre-labelled FACS tubes 25 µl of each sample was added and the same volume of assay buffer was then added to new FACS tubes for a duplicate blank reaction. The 25 µl pre-prepared Bead Mix was added to all tubes including blanks followed by 50 µl of the Biotin-Conjugate mix. Each reaction tube was gently vortexed and incubated at RT (18 – 25°C) for 2 hours away from light. Meanwhile, the Streptavidin-PE solution was prepared for a total of 94 samples, with volume allowance, by diluting 176 µl in 5324 µl of assay buffer (i.e. 1:31 dilution).

After the first incubation, 1 ml of the assay buffer was added to all FACS tubes using a repeat pipette then tubes were centrifuged for 5 min at 200 x g. To decant the supernatant, the FACS tube was quickly inverted only once. About 100 µl of residual fluid remained in the tube. This wash step was repeated again. Then 50 µl of the pre-prepared Streptavidin-PE solution was added to all tubes, vortexed to mix, and incubated for 1 hr at RT in the dark. Afterwards, the same washing procedure was repeated twice. Finally, 500 µl of assay buffer was added to each tube and protected from light. The reactions were then ready to be read on the flow cytometer, however, they could be stored at 4°C for up to 24 hours. For this experiment, the reactions were stored overnight.

The BD FACS Canto™ II flow cytometry instrument was used for the data collection. Assistance was sought from Dr Gary Warnes, (Flow Cytometry Core Facility Manager, QMUL), with the initial program set up. A linear dot plot window was opened for forward scatter (FS) (x-axis) and side scatter (SSC) (y-axis). Two more windows were opened for PE emission (around 575nm) and far red for our instrument which was in the PerCP-Cy5 channel (around 700nm). The plots were set to log mode and all compensation set to zero. Setup beads (supplied in the kit) were vortexed and 500 µl added to a new FACS tube. The FS and SSC parameters were adjusted so that the small and large beads could be visualised separately as demonstrated in the first plot in **Fig. 3.2.1.1**. Upon selecting each bead population (A and B) and having these represented in the two new scatter plots, the PE voltage was adjusted so that the beads were against the left side of the plot (see Plot 2 in **Fig 3.2.1.1**). Then the PerCP-Cy5 voltage was adjusted to clearly separate each population parallel to each other. This set up was saved for subsequent experiments.

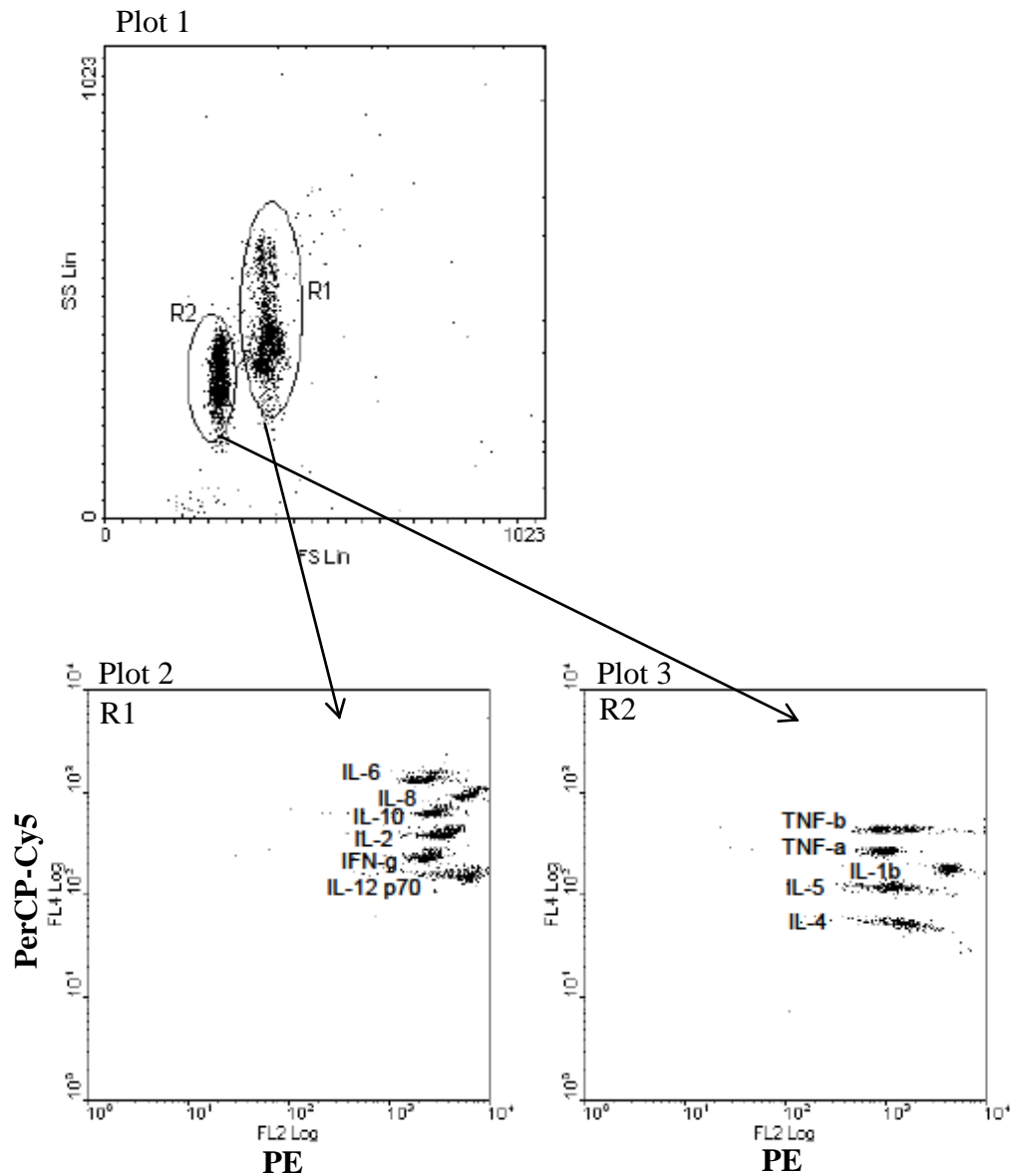


Fig. 3.2.1.1. Demonstration of bead populations for different sizes and emissions on the flow cytometer. This image shows 11 analytes from the Human Th1/Th2 11plex FlowCytomix Kit manual. The correct bead set up was critical in order to analyse the cytokines properly.

While still in Set Up mode, Standard 1, with the highest analyte concentration was run on the flow cytometer and the PE emission was adjusted to that the bead populations were as far right on the plots as allowed (**Fig 3.2.1.1**, plots 2 and 3). Afterwards, the Setup Mode was switched to Acquisition Mode and 10,000 events for each standard, blank, and sample were collected. All standards produced a measurable cytokine range with a standard curve efficiency $\geq 97\%$ except for IFN- γ which had a 94% efficiency. Analyte concentrations were calculated against the standard curves using the FlowCytomix™ Pro 3.0 Software.

3.2.1.2. The Sample Number for Saliva and Serum from Different Patient Groups

The 37 final saliva samples for each group consisted of: HC n=10, RASq n=5, RASa n=2, BDq n=11, and BDa n=9. Thirty-seven matched serum samples from the same patients who donated saliva on the day were also measured. The patients were kept in the same active or quiet category based on the presence or absence of an oral ulcer. One BDq serum sample was read erroneously on the flow cytometer so had to be excluded. Therefore the 36 final serum sample numbers for each group were: HC n=10, RASq n=5, RASa n=2, BDq n=10, and BDa n=9. Patient demographics are shown in **Table 3.2.1.2.1.**

Groups	HC	RASq	BDq	RASa	BDa
n	10	5	11*	2	9
Mean age (years)	31.6	43	37.8	34.5	40.6
Range (years)	19 - 48	25 - 59	25 - 50	26-43	21 - 57
Sex	5 M, 5 F	3 M, 2 F	6 M, 5 F	1 M, 1F	1 M, 8 F
Number of Patients Not On Any Medication	10/10 (100%)	5/5 (100%)	1/11 (0.09%)	2/2 (100%)	2/9 (22.2%)
Number of Patients Taking BD-Related Medication	n/a	n/a	6/11 (54.5%)	n/a	7/9 (77.8%)
Number of Patients using Med MWash	n/a	n/a	1/11 (0.09%)	n/a	6/9 (66.7%)

Table 3.2.1.2.1. Patient Demographics for Saliva and Serum Analysis. n, patient number. a, active/oral ulcer present. q, quiet/no ulcer present. M, male. F, female. **Med MWash**, medicated mouthwash. n/a, not applicable. *serum from one BDq patient had to be excluded due to erroneous flow cytometer reading.

3.2.1.3. Statistical Analysis

Because of the relatively small sample size, the median cytokine measurements in each group were compared to one another using the Mann Whitney U, non-parametric statistical test (2 tailed) for two independent samples. Significance is indicated by *p<0.05, **p<0.02. Welch's correction for small samples was also completed for the RASa group.

3.3. Results

3.3.1. Pro-Inflammatory Cytokines in Saliva and Serum

In order to directly compare the saliva and serum, the figures listed in **Table 3.3.1.1.** were generated by plotting the raw data from the FlowCytomix™. Therefore, this does not take into account the total protein quantity in each sample. Overall, saliva cytokines were more readily detectable than serum in 25 µl of a neat sample.

Cytokine Classification (Predominant expression)	Analyte	Assay Sensitivity* (pg/ml)	Saliva Results in Figure:	Serum Results in Figure:
Chemokine	IL-8	0.5	Fig. 3.3.1.1	Fig. 3.3.1.1
Th1	IL-1β	4.2	Fig. 3.3.1.1	Fig. 3.3.1.1
Th1	TNF-α	3.2	Fig. 3.3.1.1	Fig. 3.3.1.1
Th1	IL-2	16.4	Fig. 3.3.1.2	Fig. 3.3.1.2
Th1	IL-12 p70	1.5	Fig. 3.3.1.2	Fig. 3.3.1.2
Th1	IFN-γ	1.6	Fig. 3.3.1.2	Fig. 3.3.1.2
Th1	TNF-β	2.4	Fig. 3.3.1.3	Fig. 3.3.1.3
Th2	IL-4	20.8	Fig. 3.3.1.3	Fig. 3.3.1.3
Th2	IL-5	1.6	Fig. 3.3.1.3	Fig. 3.3.1.3
Th2	IL-6	1.2	Fig. 3.3.1.4	Fig. 3.3.1.4
Th2	IL-10	1.9	Fig. 3.3.1.4	Fig. 3.3.1.4
Th17	IL-17A	2.5	Fig. 3.3.1.4	Fig. 3.3.1.4

Table 3.3.1.1. The FlowCytomix™ Th1/Th2 Analyte Panel. The classification, assay sensitivity, and corresponding result figures for the 12 pro-inflammatory cytokines/chemokines tested in 25 µl of undiluted saliva and serum from different patient groups using a novel flow cytometry multiple bead-bound antibody assay. *The upper detectable limit was 20,000 pg/ml for each analyte except for IL-8 and IL-17A whose upper limit was 10,000 pg/ml.

Out of the 12 cytokines measured in saliva, IL-1β and IL-8 were found to be the most abundant across all patient groups (**Fig. 3.3.1.1**). The group median levels of IL-1β in HC, RASq, and BDq saliva were approximately 2 times higher than their IL-8 levels. BDa had 2.5 times the concentration of salivary IL-1β than IL-8. There was one HC whose saliva had increased levels of IL-8 and IL-1β resulting as outlying value in both graphs. HC serum did not have any detectable IL-1β and only 1 out of the 5 RASq serum samples gave a value. Both BDa and BDq serum had elevated IL-1β and IL-8. The saliva levels of TNF-α were similar amongst HC, RASq, and BDq with a group median level of 40.2, 42.2, and 42.1 pg/ml, respectively. Similarly, serum TNF-α levels were nearly equal in HC and RASq at 13.01 pg/ml and 13.95 pg/ml, however BDq serum levels were slightly raised at 37.4 pg/ml. Both patient groups with oral ulcers showed increased TNF-α.

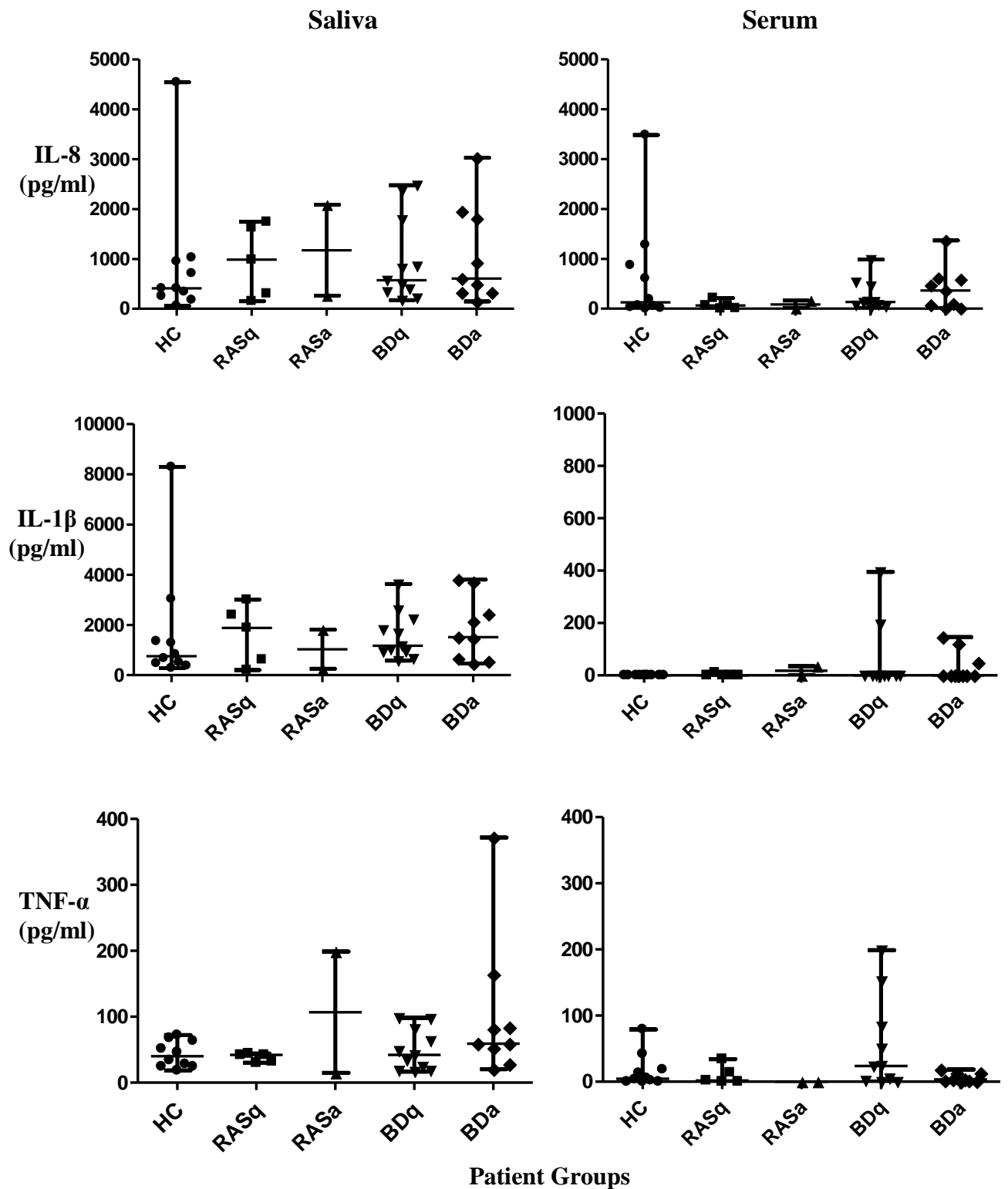


Fig. 3.3.1.1. IL-8, IL-1 β , and TNF- α concentration differences in saliva and serum from matched patients. The centre bar within each group's dot plot indicates the group median concentration. The extended bars indicate maximum and minimum values. Graphs on the left are saliva measurements and on the right are serum. Note, saliva IL-1 β y-axis is 10X the scale of serum. Patient groups include **HC** (healthy control), **RAS** (recurrent aphthous stomatitis), and **BD** (Behçet's disease). **q**, indicates mouth quiet/no ulcers. **a**, refers specifically to active mouth ulcer(s) regardless of other systemic symptoms. No significant differences between patient groups were identified.

When evaluating the Th1 cytokine panel, there was only one significant difference between the groups. Serum IL-2 was significantly increased in RASa than RASq, although the sample size was small (n=2) (**Fig. 3.3.1.2**). It was readily detected in all saliva samples and most serums. The median group values for IL-2 in saliva were similar across all groups (ranged from 138 – 190 pg/ml). The serum values for HC, RASq, and RASa were also very similar (ranged from 101 – 133 pg/ml), however the IL-2 in BDq serum was 2.5 times these levels. RASq and RASa had the highest levels of IL-12p70 in their saliva, but was not detected in their serum at all. IFN- γ in serum was also not detected.

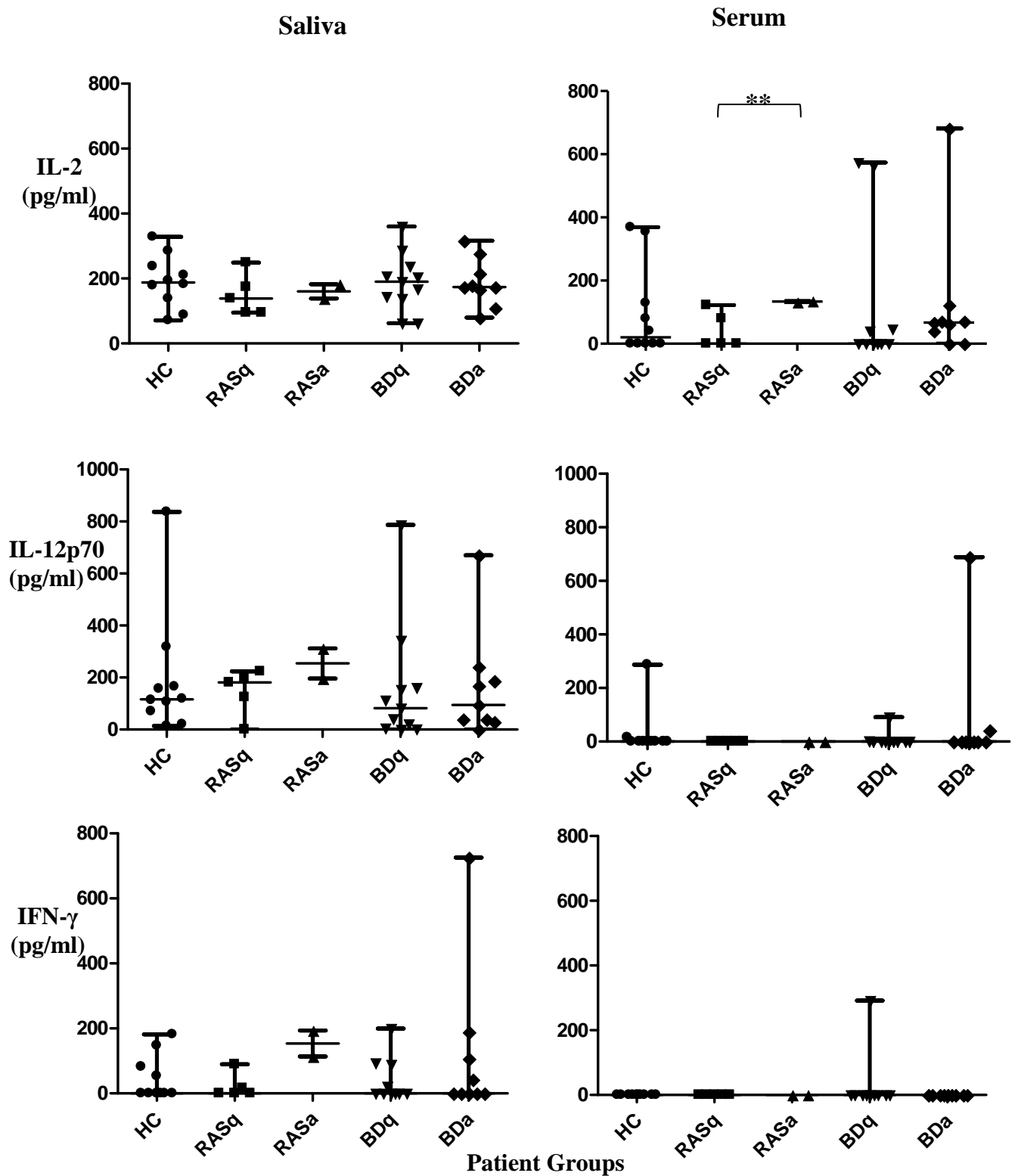


Fig. 3.3.1.2. IL-2, IL-12p70, and IFN-γ concentration differences in saliva and serum from matched patients. The centre bar within each group's dot plot indicates the group median concentration. The extended bars indicate maximum and minimum values. Left, saliva measurements. Right serum measurements. Patient groups include **HC** (healthy control), **RAS** (recurrent aphthous stomatitis), and **BD** (Behçet's disease). **q**, indicates mouth quiet/no ulcers. **a**, refers specifically to active mouth ulcer(s) regardless of other systemic symptoms. ** $p < 0.02$ (with Welch's correction).

Amongst the Th2 cytokine measurements in saliva, the majority of patient groups showed group medians below 100 pg/ml, with the exception of the RASa group. TNF- β was infrequently detected in serum samples where as in saliva more measurements were able to be identified (**Fig. 3.3.1.3**). HC, BDq, and BDa saliva shared a similar IL-4 median concentration. HC IL-4 was measured having 96.6 pg/ml while BDq and BDa shared exactly the same quantity of 93.1 pg/ml. RASq and RASa also had similar saliva IL-4 concentrations to each other at 67.1 and 61.2 pg/ml, respectively.

IL-5 had similar median saliva concentrations among groups (77.9 pg/ml) except for RASa being slightly higher at 125.7 pg/ml. In serum, RASa IL-5 was relatively low, 32.8 pg/ml, compared to the other groups which had a median of 69 pg/ml collectively.

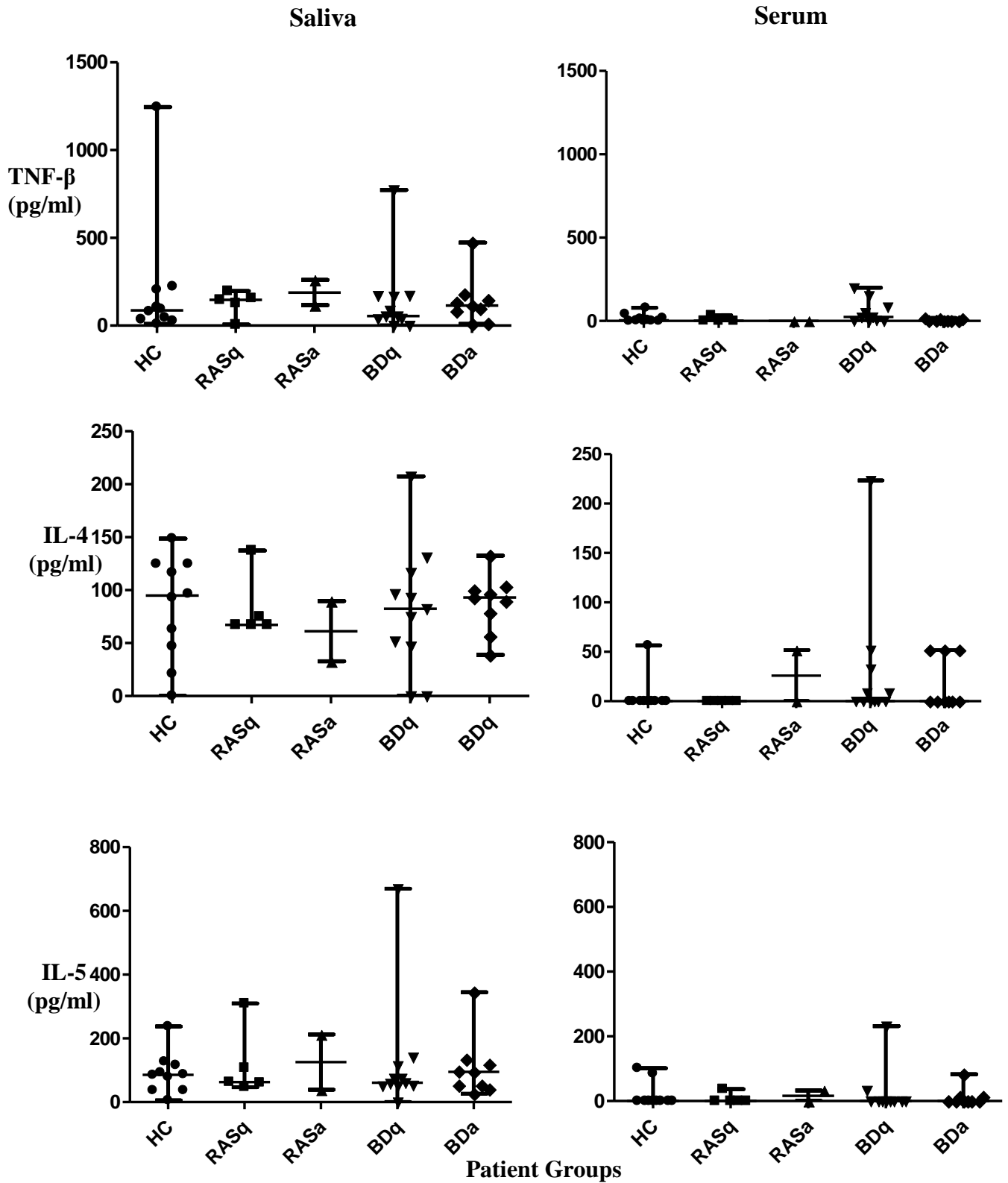


Fig. 3.3.1.3. TNF- β , IL-4, and IL-5 concentration differences in saliva and serum from matched patients. The centre bar within each group's dot plot indicates the group median concentration. The extended bars indicate maximum and minimum values. Left, saliva measurements. Right, serum measurements. Patient groups include **HC** (healthy control), **RAS** (recurrent aphthous stomatitis), and **BD** (Behçet's disease). **q**, indicates mouth quiet/no ulcers. **a**, refers specifically to active mouth ulcer(s) regardless of other systemic symptoms. No significant differences between groups were identified.

Both saliva and serum, IL-6 had the lowest concentrations across all patient groups (**Fig. 3.3.1.4**). However, significance was still able to be detected. BDa patient saliva was significantly higher than HC ($p=0.045$) and BDq ($p=0.0342$). The RAS patients with active oral ulcers also had significantly higher IL-6 levels than RAS without ulcers ($p=0.01$).

IL-10 in saliva was similar in HC and RASq groups at 50.5 pg/ml and 56.0 pg/ml, respectively. RASa had the highest median level at 169.8 pg/ml. BDq and BDa had similar levels (75.7 pg/ml and 65.9 pg/ml, respectively) with one high outlier in each group. Serum levels in HC, RASq, and RASa groups were low or undetectable. BDq only had a few measurements giving a median level of 12.5 pg/ml.

IL-17A was detected in the majority of saliva samples but not in serum. BDq and BDa IL-17A in saliva had a similar distribution and median level of 29.5 pg/ml and 27.5 pg/ml, respectively.

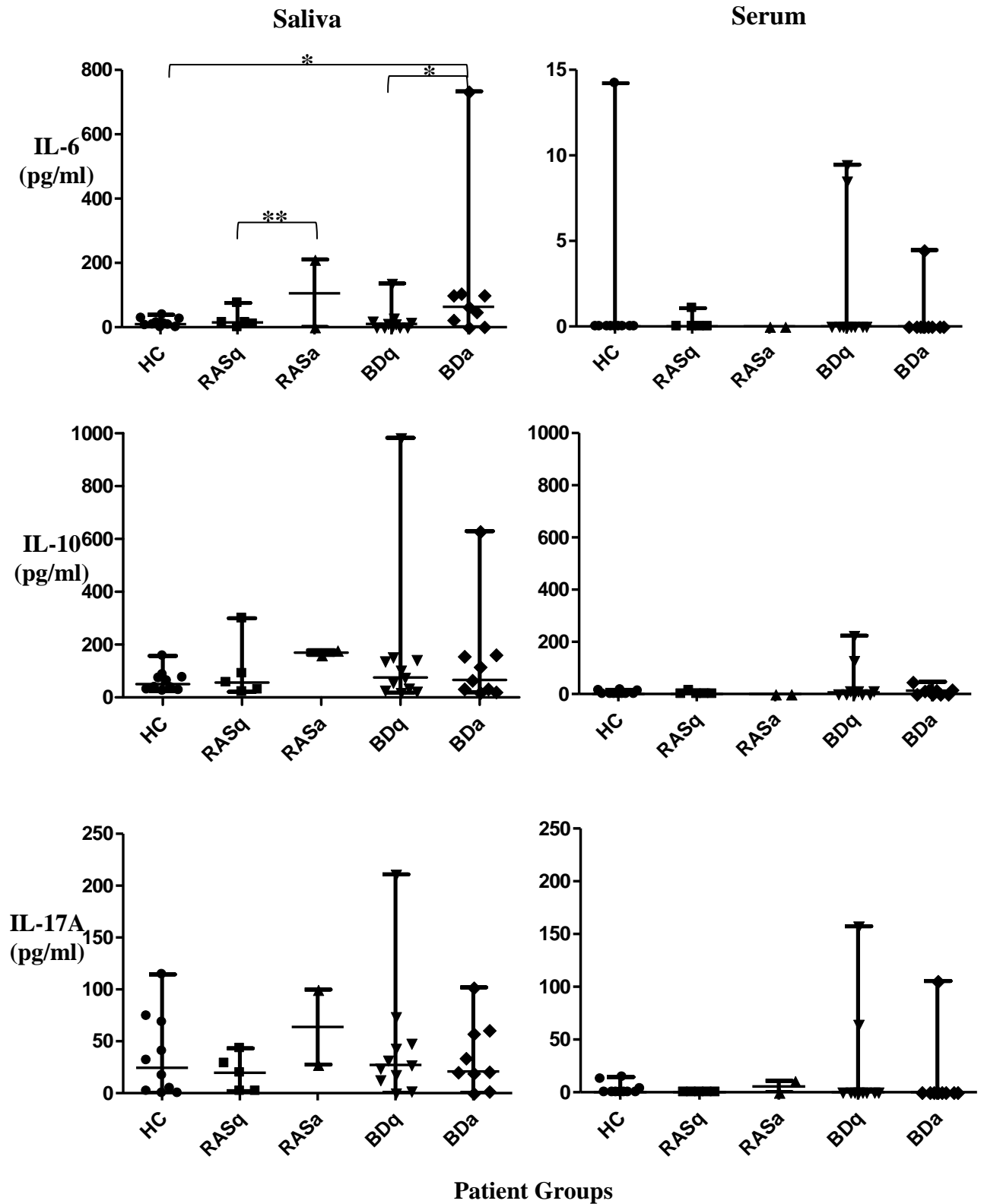


Fig. 3.3.1.4. IL-17A, IL-6, IL-10 concentration differences in saliva and serum from matched patients. The centre bar within each group's dot plot indicates the group median concentration. The extended bars indicate maximum and minimum values. Left, saliva measurements. Right, serum measurements. Patient groups include **HC** (healthy control), **RAS** (recurrent aphthous stomatitis), and **BD** (Behçet's disease). **q**, indicates mouth quiet/no ulcers. **a**, refers specifically to active mouth ulcer(s) regardless of other systemic symptoms. Note that the saliva IL-6 y-axis scale is different from IL-6 serum. Significance indicated by * $p < 0.05$, ** $p < 0.02$ (with Welch's correction).

Due to the large variability of the saliva samples, the total protein was quantified and analytically normalized based on a total protein quantification of 0.5 $\mu\text{g}/\mu\text{l}$ for each saliva sample. This concentration was chosen because it was the lowest total protein concentration quantified amongst the saliva samples. For instance, if a saliva sample's total protein concentration was 1 $\mu\text{g}/\mu\text{l}$ and its IL-8 measurement was 200 pg/ml: the designated baseline total protein (0.5 $\mu\text{g}/\mu\text{l}$) was divided by the sample's total protein quantification (1 $\mu\text{g}/\mu\text{l}$) and then multiplied by the IL-8 result for that sample (200 pg/ml). The portion of IL-8 measured amongst the total protein in saliva would be 100 pg/ml. This was conducted for each saliva sample and each cytokine measurement. As a result, some cytokine correlations were found.

The normalised saliva measurements of IL-8 and IL-1 β across all patient groups had a correlation with linear regression of 0.789 (**Fig. 3.3.1.5**). Additionally, IL-12p70 and TNF- β measured in saliva resulted in a close correlation of 0.85 (**Fig. 3.3.1.6**). Finally, IL-5 and IL-10 detected in saliva from all patient groups showed a linear regression of 0.694 (**Fig. 3.3.1.7**).

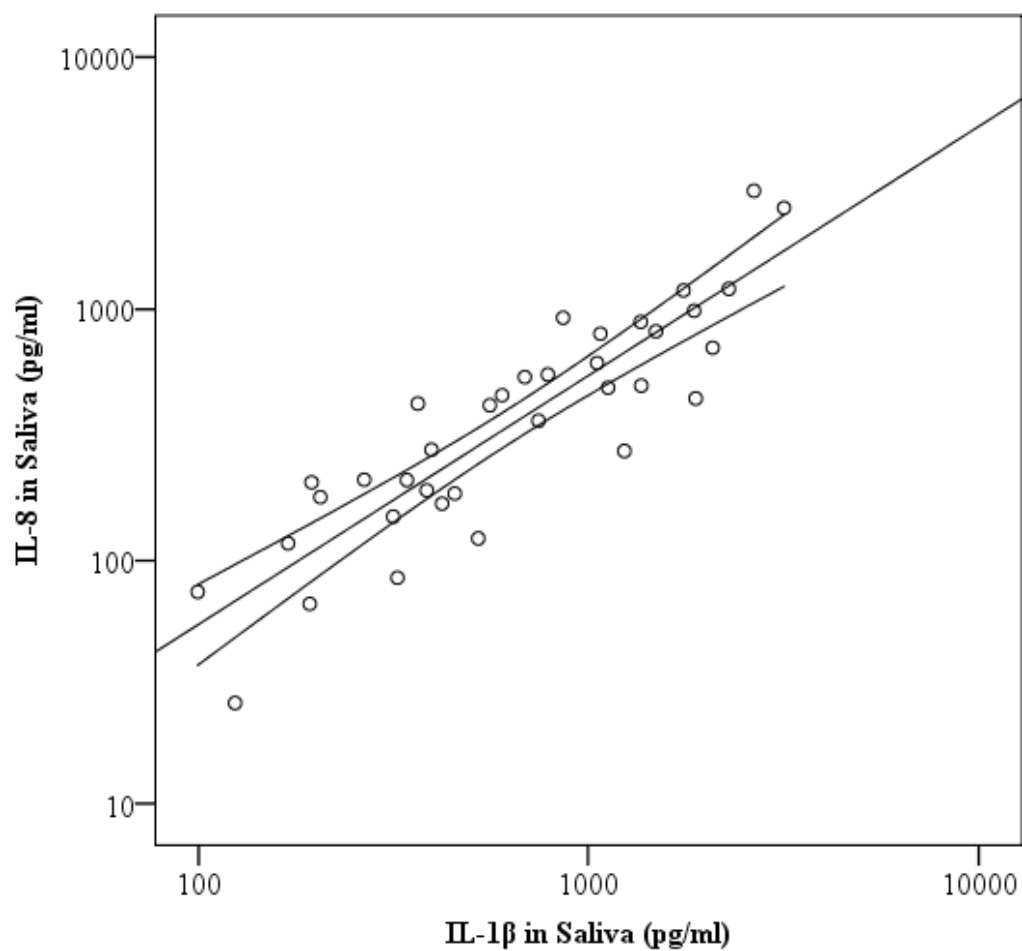


Fig. 3.3.1.5. Correlation of IL-8 and IL-1 β measured in saliva from all patient groups. Linear Regression = 0.79. Lines on either side show the 95% mean confidence intervals.

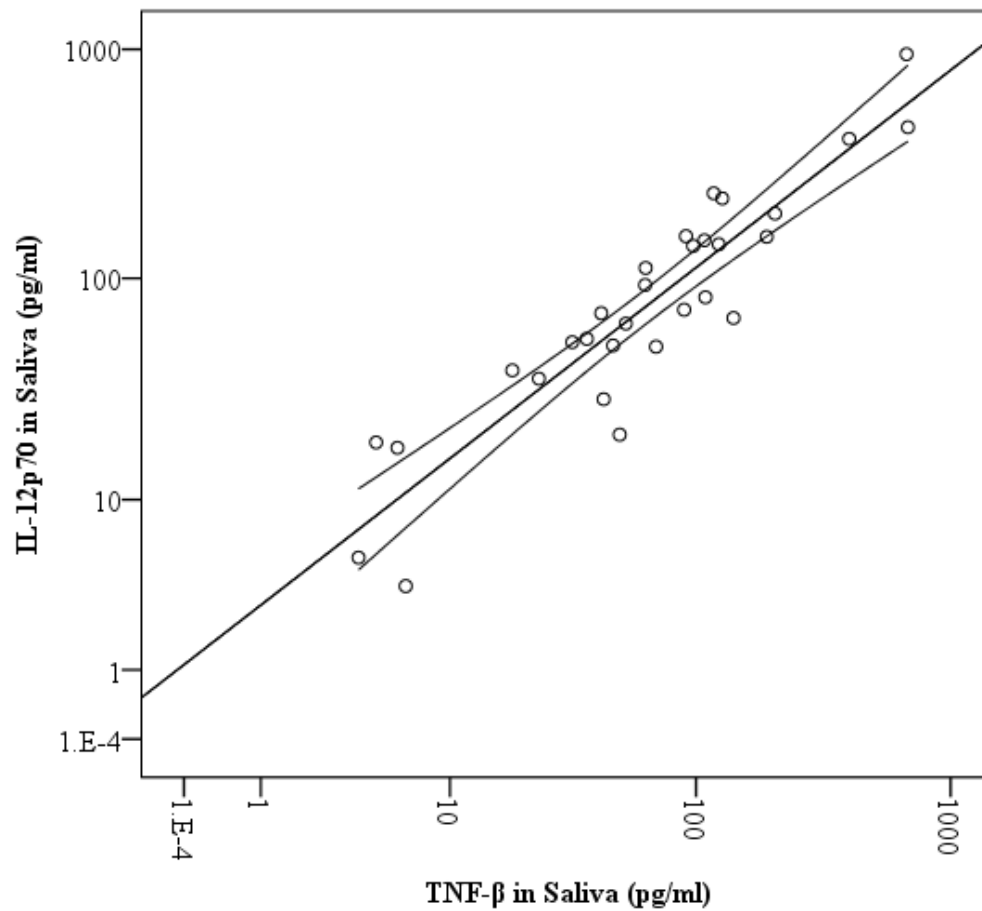


Fig. 3.3.1.6. Correlation of IL-12p70 and TNF-β measured in saliva from all patient groups. Linear Regression = 0.85. Lines on either side show the 95% mean confidence intervals.

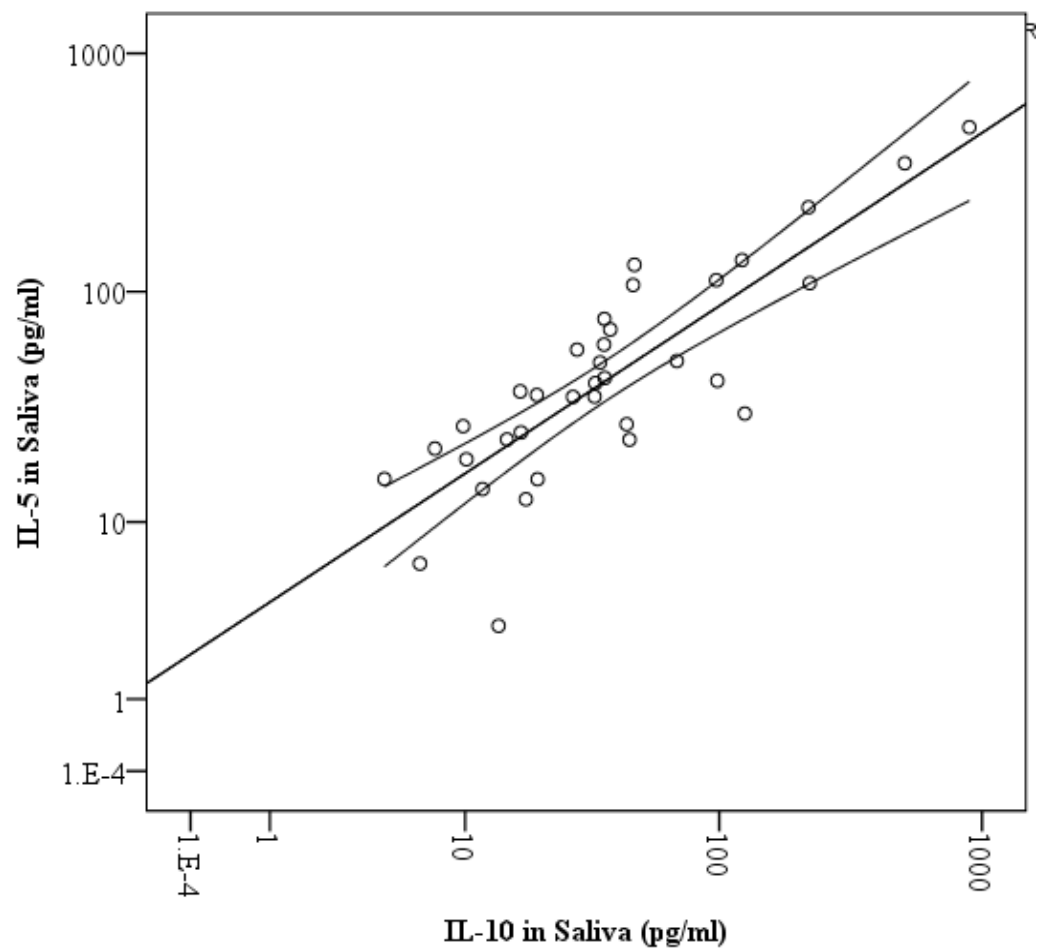


Fig. 3.3.1.7. Correlation of IL-5 and IL-10 measured in saliva from all patient groups. Linear Regression = 0.694. Lines on either side show the 95% mean confidence intervals.

3.4. Discussion

3.4.1. Saliva and Serum Inflammatory Markers

Originally, the FlowCytomix™ array was compared to a different method of cytokine measurement for saliva and plasma samples: an IL-8 ELISA (work completed by Dr Eleni Hagi-Pavli, data not shown). The assay measured IL-8 in BD plasma regardless of systemic or oral activity (BD n=50, HC n=11) and saliva supernatant samples (BD n=35, HC n=9). Mean IL-8 was found at low levels in plasma (BD $4.0 \pm \text{SEM } 1.4$ pg/ml, HC 2.1 ± 0.7 pg/ml) and high levels in saliva (BD 720.6 ± 42.36 pg/ml, HC 705.7 ± 68.4 pg/ml). In our FlowCytomix™ array, the BD patients were sub-divided depending on the presence of oral ulceration to further assess the inflammatory components of the saliva.

When measuring protein levels in saliva, it was important to analyse saliva from ulcerated mouths in a separate group for the reason that inflammatory cells and cytokines are highly likely to be at peak levels at the site of a wound. Frequently, in research studies which compare BD and RAS patients, the activity of the BD patients is well-defined, however the critical detail of whether the RAS patients were suffering an ulcer or not fails to be mentioned. This parameter needs to be better defined. Therefore, we analysed RAS saliva and serum samples from persons with (active, a) and without ulcers (quiet, q) separately and what was found was that RASa and RASq groups showed different inflammation profiles.

Some cytokine levels in RASa saliva samples were lower than expected such as in IL-1 β , but this is most likely due to the sample size being n=2. It was surprising to find that RASq had higher levels of salivary IL-8 and IL-1 β to that of HC. This suggests that IL-8 chemokine and IL-1 β in saliva may recruit inflammatory cells in RAS patients during a quiescent episode. This could provoke the onset of an ulcer. Saliva could potentially be a reliable specimen for monitoring cytokine precursors of inflammation when the mouth is non-active.

A study conducted in 1995 by Ozoran and his colleagues did not find any significant differences of the IL-8 levels in serum between BD systemically active, BD quiet, or HC (Ozoran, 1995). However, since then, a multitude of studies have reported significant findings based on ELISA assays. When the criteria for active or symptomatic systems were analysed separately, so that the sera from patients with oral ulcers were compared to

those without ulcers, IL-8 was significantly increased in individuals with oral ulcers (Zouboulis, 2000; Katsantonis, 2000; Durmazlar, 2009). Importantly, our study also found that this was the case, but in addition, also found high levels of serum IL-8 in BD quiet patients that were non-symptomatic.

Zouboulis, *et al*, also reported that up to 51% of their serum samples were below detectable limits of their ELISA assay (10 ng/ml) so that, for example, 19 BD quiet sera samples were tested but only 4 samples could detect IL-8 (Zouboulis *et al.*, 2000). Using the FlowCytomix™ array, we had an improved yield and only 2 out of 38 serum samples (5%) were below our 0.5 pg/ml IL-8 detection limit. Unfortunately, our other analyte targets in serum were not detected as readily as IL-8. Not including IL-8, the majority of undiluted serum samples were below detectable limits (28 out of 38 samples or 74%). However, overall, neat saliva samples showed better detection than serum. Saliva had an average of only 2 out of 35 saliva samples (6%) per analyte below detectable levels, the exception being saliva IFN- γ which had 22 missing values.

The ratio of salivary IL-8 and IL-1 β (average ratio of 1:2) showed a strong predictability that IL-1 β would be nearly twice as high than IL-8 in 89% of the samples. Together, they showed a correlation in the collective saliva samples from all groups. However, serum samples did not adhere to this ratio. Katsantonis, *et al*, suggested using IL-8 as a serological marker for assessing BD activity (Katsantonis, 2000). Our assessment would suggest that IL-8 alone is perhaps not specific enough seeing as both RASq saliva and BDq serum had high measurements of this chemokine. Granted, this could be alluding to the onset of an ulcer or other mucosal inflammation. However, IL-8 paired with IL-1 β measurements in saliva, could improve the validity of pre-empting an active episode. The IL-8 and IL-1 β correlation demonstrates the reliability of saliva samples to provide accurate data for the potential monitoring of inflammatory markers.

IFN- γ has been hailed as the classically identifiable Th1 cytokine, however detecting it with the FlowCytomix™ array was scarce. Only 14 out of 36 saliva samples and 1 out of 38 serum samples were measured successfully. IFN- γ was found to be increased in the saliva from BD patients with active ulcers which shows that only when the oral mucosa was damaged did the classic IFN- γ Th1 response drive forward. Frassanito, *et al*, also found increased circulating IFN- γ from only symptomatic BD patients when compared to BD quiet and HCs (Frassanito *et al.*, 1999). Another Th1-type cytokine, TNF- α , did not

seem to differ extensively in the saliva from most groups except for BDa patients with active oral ulcers which presumably was most likely beneficial in order to recruit cells to fight any pathogens associated with the wound. Although RAS active patients had a high median concentration of TNF- α in saliva, this was due to one out of the two patients skewing the distribution. More samples are required from this patient group for a more accurate assessment since it has been shown to be highly expressed in RAS oral lesions in a previous study (Natah et al., 2004). Chronically high levels of TNF- α (which could lead to an influx of neutrophils and mucosal damage) were not observed in BDq saliva, however, the same group showed the highest level in their serum. This result suggests that TNF- α may be involved in stimulating a systemic response in BD but not necessarily in the mouth.

IL-12p70, another pro-inflammatory cytokine which is known to drive the Th1 immune response by inducing IFN- γ and IL-2, was found to be increased in RASq saliva, although this did not allude to high levels of either IL-2 or IFN- γ . Instead, it did have a correlation with TNF- β in saliva. In saliva, IL-12p70 and TNF- β may work in close collaboration with each other.

IL-17A was incorporated into the array panel because it has been shown to recruit neutrophils whose proteases can cause detrimental damage to the mucosa. IL-17A has also been linked to autoimmune diseases. To date, it has not previously been measured in salivary fluid of BD or RAS patients, but has been investigated in chronic periodontitis in which it was significantly lower than HC (Ozcaka et al., 2011). A previous study was unable to measure IL-17A in the sera from BD patients using an ELISA method (Chi et al., 2008) however, we were able to detect it using the FlowCytomixTM. Our data suggest that IL-17A may be worth pursuing as a contributor to the inflammation seen in BD as excessive IL-17A could be a detrimental trigger for prolonged inflammation.

IL-6 is an alleged partner of IL-17 since it is known to be a stimulant towards Th17 cell differentiation which in turn releases IL-17A. Presumably, if IL-6 increased it is thought that IL-17A would also increase but this was not the case in our measurements as there was no correlation found. Serum IL-6 was seldom detected in the groups with the exception of two BDq samples. In contrast, salivary IL-6 was significantly higher in orally active BD and RAS patients although, again, this was not reflected in their IL-17A saliva levels. The IL-6 in an ulcerated mouth may drive a Th2-preferred pathway

stimulating B cell maturation instead of Th17 cell differentiation. An unknown stimulatory factor other than IL-6 or in conjunction with IL-6 may increase IL-17A production in the circulation. Low serum IL-6 in BD patients but high individual IL-17A measurements could also be because IL-17A is being produced by a non-Th17 type cell (such as $\gamma\delta$ T cells or NK cells) (Raifer et al., 2012) which does not depend on the IL-6 stimulatory pathway. However, these cytokines measurements will require a larger number of samples to be sure that IL-17A is not dependent on IL-6 in this system. IL-6 has previously been undetectable in BD and HC plasma using an ELISA method whose lower detection limit was 18 pg/ml (Yamakawa et al., 1996). The IL-6 detection limit for our FlowCytomix array was 1.2 pg/ml, and although evidence of the cytokine was found in serum, the highest average was in HC at 14.2 pg/ml. Therefore, our data coincides with that of other previous reports which state that serum IL-6 was absent or of particularly low levels in BD and HC. However, another more recent study by Curnow, *et al*, in 2008 found that IL-6 was significantly increased in BD patients (grouped together regardless of disease activity or site of activity) compared to RAS (again, not differentiated into oral ulcer activity) and HC (Curnow et al., 2008). They used a similar multiplex bead assay but with the constraint that the samples had to be read on a specialized machine, the Luminex 100TM. Once again, as seen with our own serum samples, many were below the level of detection: Curnow, *et al*, reported that only 18 out of 79 (23%) BD serum samples were detected, 3 out of 15 (20%) HCs, and 0 out of 20 RAS samples. Yet, IL-6 was still described by Curnow, *et al*, as one of the most abundant inflammatory cytokines amongst a panel of 10 detected in serum (Curnow, et al, 2008). In our study, 33 out of the 38 serum samples did not detect any IL-6. However, IL-6 in saliva had a better outcome being detected in 26 out of 36 samples and showing the highest levels in the BD group with active oral ulcers. From the results of our data, it is implausible to support the previous hypothesis that IL-6 has a prominent role to play in systemic inflammation in BD but it does suggest that the oral mucosa inflammatory cells release IL-6 in response to oral ulceration.

IL-2 can be influenced by IL-6 as well. IL-6 can promote T helper and cytotoxic T-cell proliferation by increasing their IL-2 secretion (Naka et al., 2002). In the serum samples, it was found that RASa had significantly higher levels of IL-2 compared to RASq patients. It was also measured in high levels in BDa serum possibly reflecting the ongoing inflammation process during oral ulceration. The IL-2 in saliva had similar levels detected in all groups. This indicates that locally detected IL-2 was not necessarily

affected by oral ulceration. The increased presence of IL-2 cytokine in the mouth seems unnecessarily high in HC and RASq patients without any obvious mucosal inflammation or ulcer to attend or react to.

IL-4 is an essential component to developing a Th2-type response. In our experiment, it was found to be highest in the BDq group's saliva. However in the serum, IL-4 failed to be detected in the majority of samples. Therefore, IL-4 may be a more prominent force driving the production of antibodies in the mouth other than systemically.

RASq saliva may have similar levels of IL-6 and IL-10 to HC due to anti-inflammatory affect. An increase in IL-10 has previously been found to promote wound healing (Peranteau et al., 2008). However, a decreased production of IL-6 in the oral mucosa is also thought to differentiate rapid, scarless oral mucosal healing from skin regeneration (Szpaderska et al., 2003). It would be useful to investigate salivary IL-6 and IL-10 *in vitro* to see in which pro- and/or anti-inflammatory capacity the cytokines are predominantly working as on RAS and BD mucosal epithelial cells.

IL-5 and IL-10 showed a strong correlation in all saliva samples. Interestingly, when BDq patients were re-categorised into a new group without any ulcers or systemic symptoms, they had the highest IL-5 and IL-10 concentrations in the saliva (data not shown). Both of these Th2-type cytokines can promote B cell proliferation and maturation leading towards a Th2 response. IL-10 can also inhibit T-cells from making IFN- γ (Romagnani, 1995). This could be the reason why non-symptomatic BD patients had their IL-10 saliva level as one of the highest of all groups while IFN- γ was one of the lowest. This inverse relationship was apparent in BD patients with ulcers as well: IL-10 and IL-5 were low while IFN- γ was remarkably high.

Despite all of the BD patients who donated saliva and serum were on immunosuppressive medication, they still showed high levels of inflammatory cytokines in their saliva. It was difficult to determine the outcome of the serum analysis since so many samples were below the level of detection especially for IFN- γ in which 37 out of 38 serum samples were undetectable. The values measured showed a wide range, especially for serum results, but can be more accurately assessed with larger sample sizes. The FlowCytomix™ is an expensive array but a copious amount of data was obtained with a single experiment and 39 paired saliva and serum samples in order to confirm the inflammatory state of persons who suffer with oral ulcers and/or BD.

3.4.2. Conclusions:

1. Saliva is a valuable, useful, and readily available specimen that can be used to investigate both the oral and systemic inflammation. Samples should be analysed separately depending on whether an ulcer is present or not.
2. FlowCytomixTM array is a sensitive technique which only requires a minimum of 25 µl of sample to obtain valuable measurements, especially when using saliva as a specimen.
3. Cytokine measurements in serum are often too low to provide conclusive data.
4. A high level of IL-8 and IL-1β in RAS patients' saliva when they do not have an ulcer may be the inflammatory instigators of developing an ulcer. Their anti-inflammatory IL-10 level which is similar to HCs may be overcome leading towards inflammation.
5. The immune response of systemically active BD patients without an episode of oral involvement can still influence the inflammatory markers found in saliva. Also, importantly, BD patients who are orally non-symptomatic still maintain underlying inflammation as is evident of the high levels of inflammatory cytokines in their saliva which warrants further investigation. A potential monitoring system for the saliva cytokines could pre-empt the onset of a multi-symptomatic episode. This knowledge could provide an alert system for topical or systemic therapeutic interventions.
6. The BD cytokine profile has shown a mixed Th1/Th2 response rather than predominantly Th1.

Chapter 4:
Neutrophil Elastase
and Its Protease
Inhibitors

4.1. Introduction

4.1.1. Neutrophil Elastase and its Main Protease Inhibitors: Secretory Leukocyte Protease Inhibitor and Alpha1-Antitrypsin

4.1.1.1. Neutrophil Elastase

Neutrophil elastase (NE) is a 29.5 kDa serine protease glycoprotein contained mainly within neutrophil primary azurophil granules. Neutrophils are the first leukocytes to arrive at sites of infection in order to contain and degrade microbes as a protective benefit to the host. Their typical behaviour is to ingest pathogens and then guide them to internalised compartments called phagosomes which fuse with primary azurophil granules containing active NE, myeloperoxidase (MPO), Cathepsin G (CatG) and Proteinase 3 (PR3), as well as antimicrobial peptides including alpha-defensins and lysozyme, and the ability to generate oxygen metabolites such as superoxide anion (O_2^-) (Korkmaz et al., 2008b, Witko-Sarsat et al., 2000, Faurschou and Borregaard, 2003). In this contained internal space, the digestive compartment, referred to as phagolysosome, the granule contents work simultaneously to degrade microbes.

The azurophil granules, also known as the peroxidase positive granules, and their contents are synthesised in immature neutrophils, specifically, the bone marrow myelocytic progenitor cells, during the early stage of granulocyte development (Takahashi et al., 1988) (Korkmaz et al., 2008b). This is when NE is produced. The protease is then stored in azurophilic granules at a high concentration of approximately 5.33 mM (Liou and Campbell, 1995) which equates to 0.157 pg/ml or 3-4 pg per neutrophil (Korkmaz et al., 2008a). Damiano, *et al*, has shown that two different azurophil peroxidase positive granule subsets exist in the neutrophil. The first type contains NE, is larger in size, has a small amount of peroxidase activity, and makes up 26% of the azurophil population. The second does not contain any NE and has a strong peroxidase activity (Damiano et al., 1988).

4.1.1.2. Neutrophil Granules, NE and Exocytosis

As well as acting as phagocytes to ingest and digest microbes, viable neutrophils can also simultaneously exocytose their granule contents under controlled conditions to further induce the immune response (Lacy, 2006). Necrotic neutrophils uncontrollably release all cell contents whereas NETosis is a programmed cell death in which neutrophils purposely

externalise their DNA and azurophil contents (NETosis will be explored further in **Chapter 5**). Alongside azurophils, neutrophils also contain three other granules referred to as specific, gelatinase, and secretory vesicles. The specific (secondary) and gelatinase (tertiary) granules are also called the peroxidase negative granules (Faurischou et al., 2002, Sengelov et al., 1993). All three are synthesised at later stages of neutrophil development. Interestingly, the later the granules are produced, such as the secretory vesicles, the first they are to be released from the neutrophil. The fact that azurophils, containing potent proteases that could cause host tissue damage, are the last to be exocytosed suggests a self-regulatory mechanism. Most granules proteins have the capacity to be bactericidal, but can also help lead the migration of neutrophils. For instance, when the secretory vesicle is exocytosed, its various protein receptors incorporate themselves onto the neutrophil membrane. This enables the cell's attachment to vascular endothelial cells and its migration through vessels and tissues towards the site of inflammation (Faurischou and Borregaard, 2003, Faurischou et al., 2002). Therefore, it is fitting that secretory vesicles are the first to be exocytosed from the neutrophil in order to establish contact.

Depending on the type of stimulus, neutrophils can purposely exocytose a portion of their granules in order to instigate or propagate the immune response. For instance, an increased uptake of calcium (Ca^{2+}) induces the orderly granule release mentioned previously with azurophils being mobilised last (Sengelov et al., 1993). *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), a bacteria wall derived chemoattractant, mobilises a quarter of neutrophil's gelatinase granules but very few azurophil and specific granules (Kjeldsen et al., 1999). Specifically, $\text{TNF-}\alpha$, IL-8, complement 5a (C5a), and LPS primes neutrophils and causes NE to be released (Lee and Downey, 2001).

Granules are mainly reserved for the intracellular fusion with a phagosome containing an engulfed microorganism (Sengelov et al., 1995). However, externally shed NE participates in several roles as a propagator and suppressor of the immune system. NE has the ability to degrade various connective tissues, including extracellular matrix (ECM) components such as elastin, collagen, fibronectin, laminin, and proteoglycan (Roghianian and Sallenave, 2008a) (Heutinck et al., 2010) compromising the integrity of healthy tissue. Multiple studies have demonstrated the tissue damage caused by NE. These studies will be further reviewed later in this chapter (section **4.1.1.6**). However, if regulated appropriately, a small amount of NE tissue degradation can encourage the regeneration of

new cells. NE is also able to degrade IL-1 β , TNF- α (Owen et al., 1997), IL-2 (Ariel et al., 1998), and IL-6 (Bank et al., 1999) inflammatory cytokines thereby downgrading the response. NE can also cleave CD14 which is the main receptor for bacterial LPS on blood monocytes and gingival fibroblasts (Le-Barillec et al., 1999, Nemoto et al., 2000).

Despite the damage that NE can cause, neutrophils do take on a very important and helpful role in the reparative processes by phagocytising debris and keeping a local area clear so that additional neutrophils and other cellular components and pro-inflammatory cytokines can gain access to an infected area (Ghasemlou et al., 2010). Neutrophils are an essential part of this process. With a low number or absence of neutrophils, the innate immune response is stunted and individuals can develop severe, recurrent infections and may end up suffering from mouth ulcers (Bouma et al., 2010). A delicate balance of neutrophils and NE is paramount for normal tissue function.

The life span of circulating neutrophils has been debated for many years. In 1997, neutrophils were estimated to have a half-life of around 6 hours (Haslett, 1997). This was revised in 2000 by Webb, *et al*, with the realisation that they could live for 24-48 hours (Webb et al., 2000). More recently, in 2010, Pillay, *et al*, showed that HC neutrophils can circulate *in vivo* for 5.4 days in peripheral blood (Pillay et al., 2010). However, when recruited to sites of inflammation or infection within tissues, it is believed that neutrophils can survive for longer periods by delaying apoptosis (Wright et al., 2010). Therefore, one can envisage that during chronic inflammation the presence of activated neutrophils in tissues may increase the risk of NE-related damage. Thus persistent, active neutrophils releasing NE in the oral cavity may disrupt the mucosal lining causing a type of pathology which manifests as an ulcer.

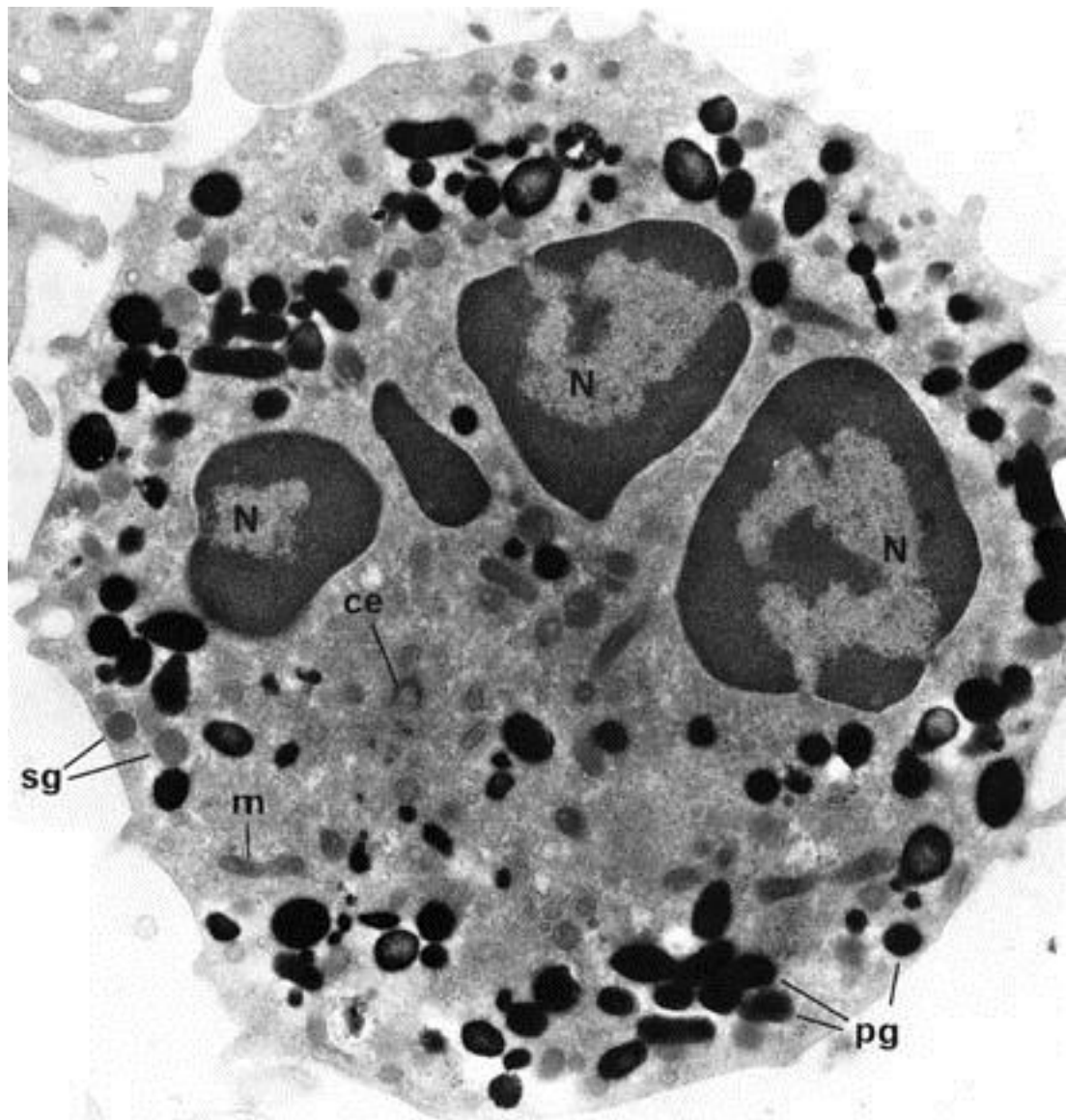


Fig. 4.1.1.2.1. The Neutrophil and Azurophil Granules. A resting neutrophil image taken with an electron microscope showing the azurophil granules (or primary granules, pg), which appear as large dark granules. Secretory granules are also indicated (sg). Other features that are indicated are the nucleus (N); centriole (ce); mitochondri (m). Reproduced with permission from Witko-Sarsat, *et al*, 2000 (Witko-Sarsat et al., 2000).

4.1.1.3. Secretory Leukocyte Protease Inhibitor

Secretory leukocyte protease inhibitor (SLPI) is a small 11.7 kDa stable innate inhibitory protein which has broad spectrum anti-bacterial, anti-fungal, and anti-viral activity and protects mucosal epithelial tissue surfaces from endogenous proteolytic enzymes (serine proteases) such as trypsin, cathepsin G, and neutrophil elastase (NE) (Jacobsen et al., 2008). SLPI has been found in various secretions including seminal plasma (Ohlsson et al., 1995), cervical mucus (Moriyama et al., 1999), nasal (Lee et al., 1993), and bronchial secretions (Sallenave, 2000), sputum (Sallenave et al., 1997), reflex tears in the eye (Sathe et al., 1998), and saliva stemming from the parotid gland (Thompson and Ohlsson, 1986). It is produced in the non-ciliated epithelial cells of the lung mucosa (De Water et al., 1986), cultured immortalised oral buccal mucosa epithelial cells from stillborn foetus (Jana et al., 2005), alveolar macrophages (Sallenave, 2000), and pancreatic B-cells (in the islets of Langerhan cells) (Nystrom et al., 1999).

SLPI is also secreted from oral buccal epithelial cells (Nittayananta et al., 2013b) and salivary gland acinar cells (Ohlsson et al., 1984) and therefore is considered a local inhibitor of serine proteases NE and CatG in the oral cavity. SLPI can also be found in the specific (secondary) granules of human neutrophils (Sallenave et al., 1997, Jacobsen et al., 2008). It has been reported to be the primary regulatory inhibitor of human NE in sputum supernatants of Chronic Obstructive Pulmonary Disease (COPD) patients (Piccioni et al., 1992) and has been shown to contribute to oral mucosa wound healing in mice (Angelov et al., 2004).

4.1.1.4. Alpha1-Antitrypsin Inhibitor

Alpha1-antitrypsin (α 1AT), also known as alpha1-protease inhibitor (α 1-PI), is a 52 kDa glycoprotein which is categorised as a member of the serpin family. It is one of the best described NE inhibitors. Hepatocytes are the main producers of the protein which is then secreted into the plasma and circulated. To a lesser degree, but still important sources, α 1AT mRNA has also been found in breast milk macrophages (Perlmutter et al., 1985), alveolar macrophages (Mornex et al., 1986, van 't Wout et al., 2012), blood monocytes (Isaacson et al., 1981, Mornex et al., 1986, Perlmutter et al., 1985), monocyte-derived dendritic cells (van 't Wout et al., 2012), human lung epithelial cells (Cichy et al., 1997) and various lung cell lines, such as the A549 cells (Morgan et al., 2009), but not in blood

lymphocytes (Mornex et al., 1986). α 1AT has been detected in neutrophil azurophil granules (du Bois et al., 1991, Mason et al., 1991), and was recently found in fractionated portions of neutrophils' specific granules, gelatinase granules, and secretory vesicles (Clemmensen et al., 2011).

4.1.1.5. Oral Mucosa Inflammation, Neutrophil Elastase and Regulation

In a normal healthy mouth, inflammation along the soft mucosal linings of the oral cavity is usually due to minor wounds and/or microorganisms whether they are commensal, opportunistic, or pathogenic (Marsh, 2012) (Walker, 2004). Bacteria is always present in the mouth, however if they become overgrown or invasive, the immune system increases its defences to keep them in balance. Microbial antigens such as lipopolysaccharide (LPS) can stimulate the production and release of the pro-inflammatory cytokine IL-1 β by keratinocytes, fibroblasts, endothelial cells, and leukocytes particularly monocytes which have migrated through the mucosa (Tsai et al., 1995) (Sabroe and Whyte, 2007). C5a peptides from serum and local macrophages also promote an inflammatory response from neutrophils and other leukocytes (Manthey et al., 2009). Along with IL-1 β , TNF- α is released by endothelial cells and macrophages (Feldmann and Maini, 2001). This can induce IL-8 chemokine production (Silva et al., 2009) in a variety of cells including neutrophils, macrophages, endothelial and epithelial cells. IL-8 then recruits and guides additional neutrophils toward the source of infection or inflammation (Kaburaki et al., 2003). Neutrophils externalise some NE to aid in the further up-regulation of leukocytes and contribute to the inflammatory process by recruiting more neutrophils which also produce additional IL-8 and TNF- α .

Excessive NE can cause epithelial tissue damage (Ginzberg et al., 2001). SLPI, generated from saliva glands and epithelial cells due to stimulation by bacterial LPS and human host's IL-1, TNF, and NE (Sallenave, 2000), attempts to balance the protease activity. Serum-derived α 1AT is also present having gained access to the oral cavity through the gingival sulcus along with neutrophils (Nakamura-Minami et al., 2003, Pederson et al., 1995). Both endogenous SLPI and α 1AT are found in neutrophil granules as well but it is unknown to what extent they contribute to the protease-antiprotease balance in the oral cavity. In certain cases, it is not always clear what is instigating the inflammation or if it is derived from foreign or self-antigens. Nevertheless, during chronic oral inflammation, there is an on-going cycle of these pro-inflammatory events. In a summarized illustration,

Figure 4.1.1.5.1 depicts SLPI and α 1AT's regulation of NE and their interaction with the oral epithelium and surrounding inflammatory response. NE itself is not necessarily an absolute prerequisite for initiating the inflammatory response (Hirche et al., 2004), however its persistence influences the cyclic nature of chronic inflammation (Kessenbrock et al., 2011).

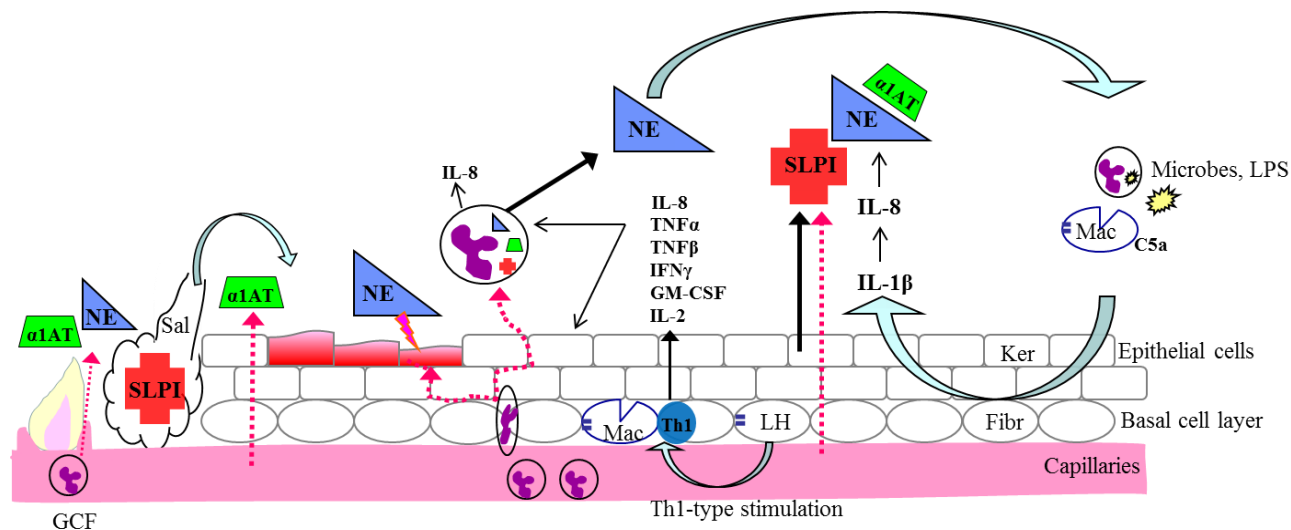


Fig. 4.1.1.5.1. Proposed Working Model of the Oral Environment During Immune Up-Regulation: Summary of the Interaction of the Epithelium, Inflammatory Cytokines, Neutrophil Elastase, SLPI and α 1AT. Microbes stimulate oral keratinocytes (Ker) and immune cells such as Langerhan cells (LH) to produce IL-8. IL-1 β can be synthesised and released by local fibroblast (Fibr) or leukocytes that have travelled through the mucosa from the vasculature (Tsai et al., 1995). IL-1 β induces further IL-8 production (Silva et al., 2009) which is a neutrophil chemoattractant (Kaburaki et al., 2003) and they migrate through the epithelium towards the site of infection and inflammation. Neutrophils phagocytose foreign entities and also release NE (and Pr3, CatG) into the extracellular environment due to continued bacterial stimulation, and presence of TNF- α , IL-8, and C5a. Neutrophil apoptosis shortly after but if they become necrotic (or undergo NETosis), they may release additional NE. Epithelial cells and salivary glands produce SLPI while GCF/serum-derived α 1AT help to regulate NE. Neutrophils also have endogenous α 1AT and SLPI but the extent of their external inhibitory contribution is not known. In addition, macrophages (Mac) and LH cells are both APC's and have MHC class II molecules. These can prime Type 1 T helper cells (Th1) (Barrett et al., 1996) to release IL-2 which induces further T-cell proliferation and additional inflammatory cytokines such as TNF- α , TNF- β , IFN- γ , and GM-CSF (Walker, 2004). TNF- α can further stimulate IL-8 production and recruit more neutrophils. Activated neutrophils can release IL-8 as well. Excessive, prolonged exposure to NE can damage cells and the integrity of the mucosal lining possibly resulting in an ulcer. (Th2 interactions are not depicted in the diagram but involve secretory IgA, IL-4, IL-6, and IL-10).

4.1.1.6. NE, Disease, and Ulceration

The continuous presence of NE in chronically inflamed tissue of individuals with chronic obstructive pulmonary disease (COPD), cystic fibrosis, and inflammatory bowel disease has been implicated as a cause of tissue damage (Stockley, 2002, Quinn et al., 2010, Heutinck et al., 2010). Excessive activity or an imbalance of NE could lead to increased pathology, auto-immunity, allergy, and tumour metastasis. Neutrophil degranulation can be triggered by various inflammatory factors such as lipopolysaccharide (LPS), TNF- α , G-CSF, C5a, and contact with ECM or cell surfaces (Nathan, 2006). Its increased presence leads to inflammation and ECM remodelling (Heutinck et al., 2010) and has been shown to cause apoptosis in intestinal (Ginzberg et al., 2004) and lung epithelial cells (Suzuki et al., 2005). Neutrophil's externalised reactive oxygen species (ROS) are known to damage cells as well but a study conducted by Smedly, *et al*, was able to show that human microvascular endothelial cells co-cultured with neutrophils from a Chronic Granulomatous Disease (CGD) patient whose neutrophils do not have the ability to produce superoxide molecules were still able to cause cellular injuries similar to that of normal HC neutrophils. Notably, the majority of the damage was repressed by a synthetic, specific NE chloromethyl ketone-type inhibitor (Smedly et al., 1986) indicating that neutrophil NE was largely responsible for the cell destruction.

Although a genetic deficiency of SLPI has not yet been reported, there are cases in which a single point mutation causes misfolding of the α 1AT protein in liver cells causing a rare disease called α 1AT deficiency. The misshapen protein accumulates in the hepatocytes instead of being released into the plasma circulation thus depleting the enzyme's availability. Eventually, the accumulation of the ill-fated protein causes liver cirrhosis, but the condition can also lead to serious lung degeneration such as emphysema/COPD due to NE in the lungs not being regulated effectively (Greene and McElvaney, 2010).

The diagnostic classification for BD, a multi-systemic inflammatory disorder of unknown aetiology, was re-visited in 2013 by an international collaboration. They maintained that oral ulcers are still the most common and reoccurring feature suffered by patients (Davatchi et al., 2013). In 1975, Matsumura and Mizushima found that neutrophil chemotaxis was evident in BD (Matsumura and Mizushima, 1975) which sparked further neutrophil-based investigations. Neutrophil infiltration is evident in oral, ocular and cutaneous BD lesions and has been implicated in the disease's progression and pathology

(Verity et al., 2003). In advanced eye involvement, a hypopyon, which is a pooled cluster of white blood cells including neutrophils, can occur in the eye and is characteristic in BD. Although the hypopyons usually disappear, the person is at high risk of future blindness (Kaklamani et al., 1998). The human leukocyte antigen (HLA) gene locus subtype B51 was also found to have an association with neutrophil hyperactivity (O'Duffy et al., 1983, Sensi et al., 1991). Since these early investigations, most studies on neutrophils and their association with BD have been solely based on blood samples. However, investigating salivary NE content may help elucidate what is occurring in these patients since neutrophils are the most common leukocytes normally found in saliva (Walker, 2004). If individuals with BD are not able to regulate the influx of neutrophils and the presence of extracellular NE in their oral cavity, perhaps the mucosa is overexposed to the proteolytic enzymes generated by neutrophils thus playing a part in their recurrent ulcer pathology.

Recurrent aphthous oral ulcers are also a feature of numerous immune dysregulations such as Crohn's Disease (Fatahzadeh et al., 2009), systemic lupus erythematosus (Munoz-Corcuera et al., 2009), oral lichen planus (Munoz-Corcuera et al., 2009) but can also arise from radiation and chemotherapy (Sonis, 2004), and even severe stress. Individuals with recurrent aphthous stomatitis (RAS), who harbour no other obvious systemic features of immunopathology, are thought to endure a unique immune dysregulation which, for unknown reasons, manifests solely in the oral cavity (Natah et al., 2004).

Ueta, *et al*, has suggested that individuals with RAS may harbour an immune cell dysfunction based on the evidence that their neutrophils had suppressed superoxide production compared to HCs, however, this was based on blood neutrophil investigations (Ueta et al., 1993). However, as is the case with BD, the majority of RAS investigations are based on peripheral blood neutrophils as opposed to those found in saliva. According to work completed by Lakschevitz, *et al*, even HC oral and peripheral blood neutrophils behave differently. Based on microarray data, they found that HC saliva neutrophils RNA changed their transcriptome when they migrated from the blood to the oral environment (Lakschevitz et al., 2013). This alteration was especially evident during increased oral infection or inflammation such as in periodontal disease where oral neutrophils showed delayed apoptosis leading to extended activity in the mouth (Lakschevitz et al., 2013). It has also been suggested that neutrophils are already primed by chemotactic factors such as IL-8 or TNF- α by the time they reach the oral cavity (Nakahara et al., 1998). Although

some aspects of salivary neutrophil function, such as the reduced phagocytosis during acute ulceration, have been explored in RAS (Lukac et al., 2003), the role of neutrophil elastase and its inhibitors have not yet been elucidated. Also, many previous studies frequently overlook sampling from RAS patients who do not have an ulcer or in other words are in a “quiet/quiescent” state. However, this is a very important group to investigate so that their typical state of immunity can be better understood.

As a disease biomarker, NE levels may lack specificity (Heutinck et al., 2010), for instance, increased NE is found in the lungs of individuals with COPD (Stockley, 2002), but can also be found in large quantities at most tissue damaged sites since neutrophils will attend to wounds in order to combat invading microbes. However, investigating NE in combination with its innate regulatory factors, SLPI and α 1AT, can help to provide a clearer understanding of their dual role in the progression of pathology since the regulation of NE may be key to understanding mucosal tissue damage in BD. It has been shown that SLPI dominates over α 1-AT concentrations in sputum (Moreau et al., 2008). Similarly, in saliva, α 1-AT has been found in significantly lower concentrations than SLPI (Cox et al., 2006) therefore SLPI is thought to be the main NE inhibitor in these biological fluids. Also, α 1-AT does not completely inhibit elastin-bound NE *in vitro* (Morrison et al., 1990). The mucosal pathology seen in BD could involve α 1AT and/or SLPI not being able to regulate NE effectively. In this study, it is hypothesised that an inadequate production or availability of SLPI and/or α 1AT in the mucosal membranes and saliva of RAS and BD patients could perpetuate NE damage contributing to recurrent oral ulcerations.

4.1.1.7. Theories on the Mechanism by which SLPI and α 1AT Regulate NE: Forming Complexes

It has been suggested that SLPI-NE complexes exist in lung epithelial cells *in vitro* (Sullivan et al., 2008). Stanley, *et al*, have suggested that the same complex is present *in vivo* in sputum supernatants from bronchiectasis patients (Chan et al., 2003). SLPI was shown to completely inhibit extracellular elastin-bound NE *in vitro* (Morrison et al., 1990) or ECM-bound NE but has been described as being reversible particularly in the lungs where it is thought that α 1-AT picks up the NE from the SLPI complex (Bingle and Tetley, 1996). The serpin α 1-AT is described as being able to disassociate (or reverse) the SLPI-NE formation (Gauthier et al., 1982) and in turn the larger α 1-AT protein traps NE

into an irreversible complex (Carrell et al., 1982). A proposal that SLPI is recycled has also been brought forth (Bingle and Tetley, 1996). This is supported by the general information on canonical inhibitors (such as SLPI) which theorises that its target proteases (such as NE) are complexed into tight, non-covalent interactions rendering the protease inactive by directly blocking its substrate active site (Krowarsch et al., 2003) (Moreau et al., 2008). It is thought that this newly formed structure can be disassembled because the inhibitor (SLPI) does not undergo conformational changes whereas the serpin-type inhibitor (α 1-AT) does (Krowarsch et al., 2003). In Koizumi, *et al*'s, structural model of NE and the active carboxyl (C-)terminal of SLPI, 6 hydrogen bonds were found between the enzyme and inhibitor (Koizumi et al., 2008). These hydrogen bonds were sufficient to inhibit NE, but hydrogen bonds are weaker than ionic or covalent bonds. Therefore, their experiment further supported the theory that the NE:SLPI complex could be reversed.

As briefly mentioned, there are different sites on the SLPI protein which are specifically attributed to its various inhibitory interactions. For instance, the anti-microbial and anti-inflammatory capabilities are thought to be controlled by the amino (NH₂ or N-) terminal (Hiemstra et al., 1996) coupled with SLPI's strong cationic nature while the carboxyl (COOH or C-) terminal is associated with limiting protease activity (Doumas et al., 2005). Although the N-terminal doesn't seem to have any anti-protease properties, it may assist with stabilizing the SLPI:NE complex (Doumas et al., 2005). The exact mechanism by which SLPI's inhibits is unknown. However, what has been shown is that the primary binding loop for NE contact and inactivation occurs between Leu72 and Met73 on the SLPI protein (Grutter et al., 1988, Koizumi et al., 2008) and is sensitive to oxidation (Carp and Janoff, 1980). SLPI:NE specificity is thought to be due to the Tyr68 residue on SLPI (Koizumi et al., 2008). It is speculated that simultaneous interactions (anti-microbial and anti-protease binding or multiple protease binding at different sites as with trypsin and chymotrypsin) can occur on a single SLPI protein (Grutter et al., 1988) (**Fig. 4.1.1.7.1**). This makes SLPI an importantly diverse, multitasking regulator of the immune system.

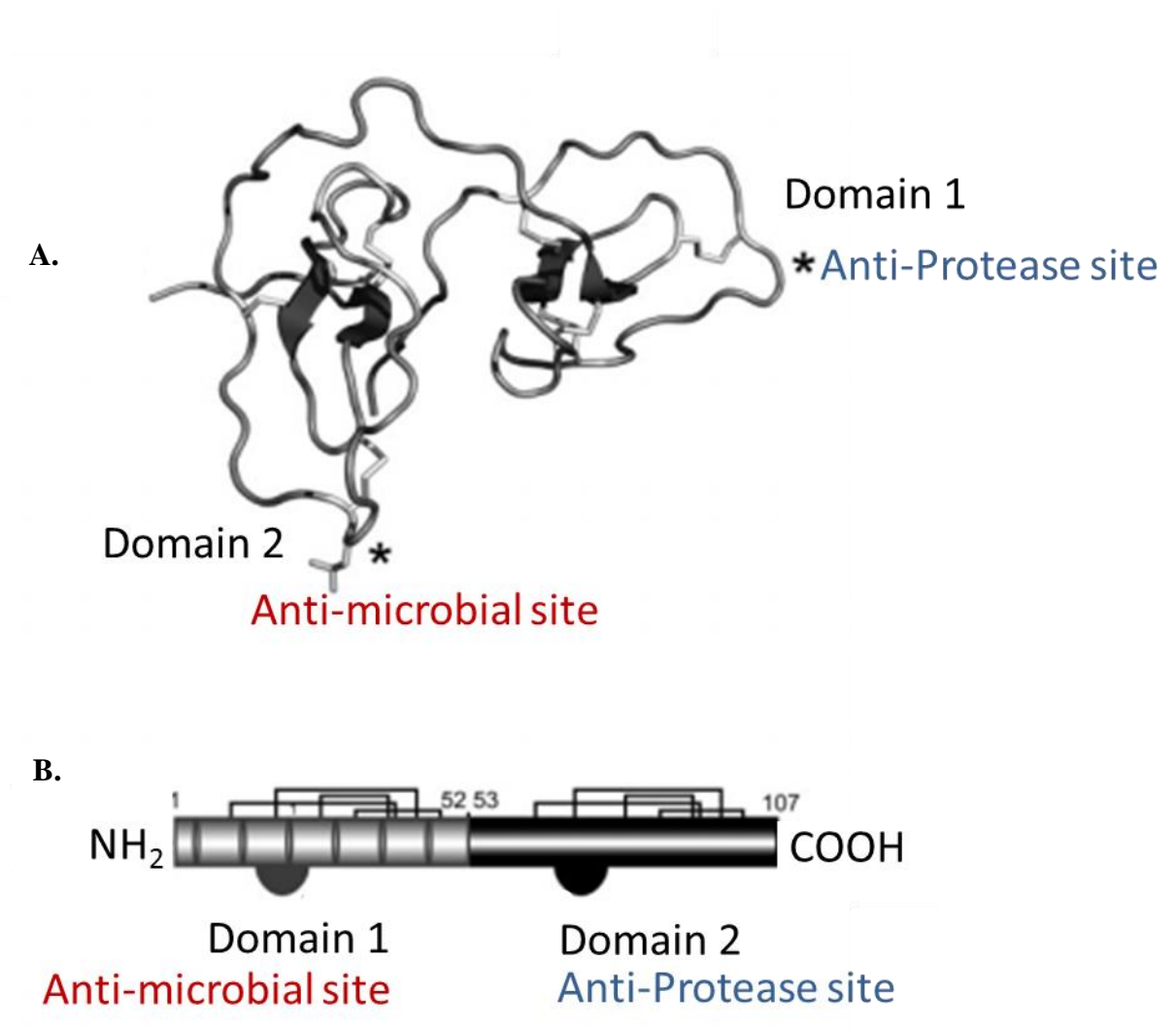


Fig 4.1.1.7.1. The Structure of SLPI Protein. The simplified structure of SLPI is shown indicating its 2 basic domains and associated activity: *indicates the reactive sites of domain 1 being anti-protease and domain 2 as the anti-microbial site. Its separate active sites may be able to inhibit proteases and microbes simultaneously. B. A schematic showing the linear configuration of domain 1, also referred to as the NH₂-terminal and domain 2, the COOH-terminal. The connecting horizontal bars indicate the four disulfide bonds in each WAP domain creating a very stable structure. Images reproduced with permission from Zani, *et al*, 2009 (Zani et al., 2009).

Alpha1-Antitrypsin has a reactive centre loop (RCL) which is located on a methionine residue at position 358 which is 37 residues from the C-terminus (Carrell et al., 1982, Dickens and Lomas, 2011). This RCL acts as a pseudosubstrate for the active site of proteinases, such as NE, so that there is a 1:1 bond of inhibitor and target. Once the protease is bound, it is moved from one end of the α 1AT molecule to the opposite end by way of the RCL being cleaved and inserting itself amongst the β -sheets as demonstrated in **Figure 4.1.1.7.2** thus trapping the protease in a covalent bond (Carrell et al., 1982, Gavrin et al., 2012b, Korkmaz et al., 2008b). Usually, phagocytic cells then clear away the inactive complex. Initially, there was doubt that α 1AT was a specific inhibitor for NE since valine or alanine are preferred elastase cleavage sites as opposed to methionine. However, there were two main indications for α 1AT being defined as the primary inhibitor for NE. The first was that its rate at which it acted to inhibit NE was faster than all its other enzymes targets (such as chymotrypsin and Cathepsin G). Second, was the fact that α 1AT-deficient models lost lung elasticity due to unregulated elastase (Carrell et al., 1982).

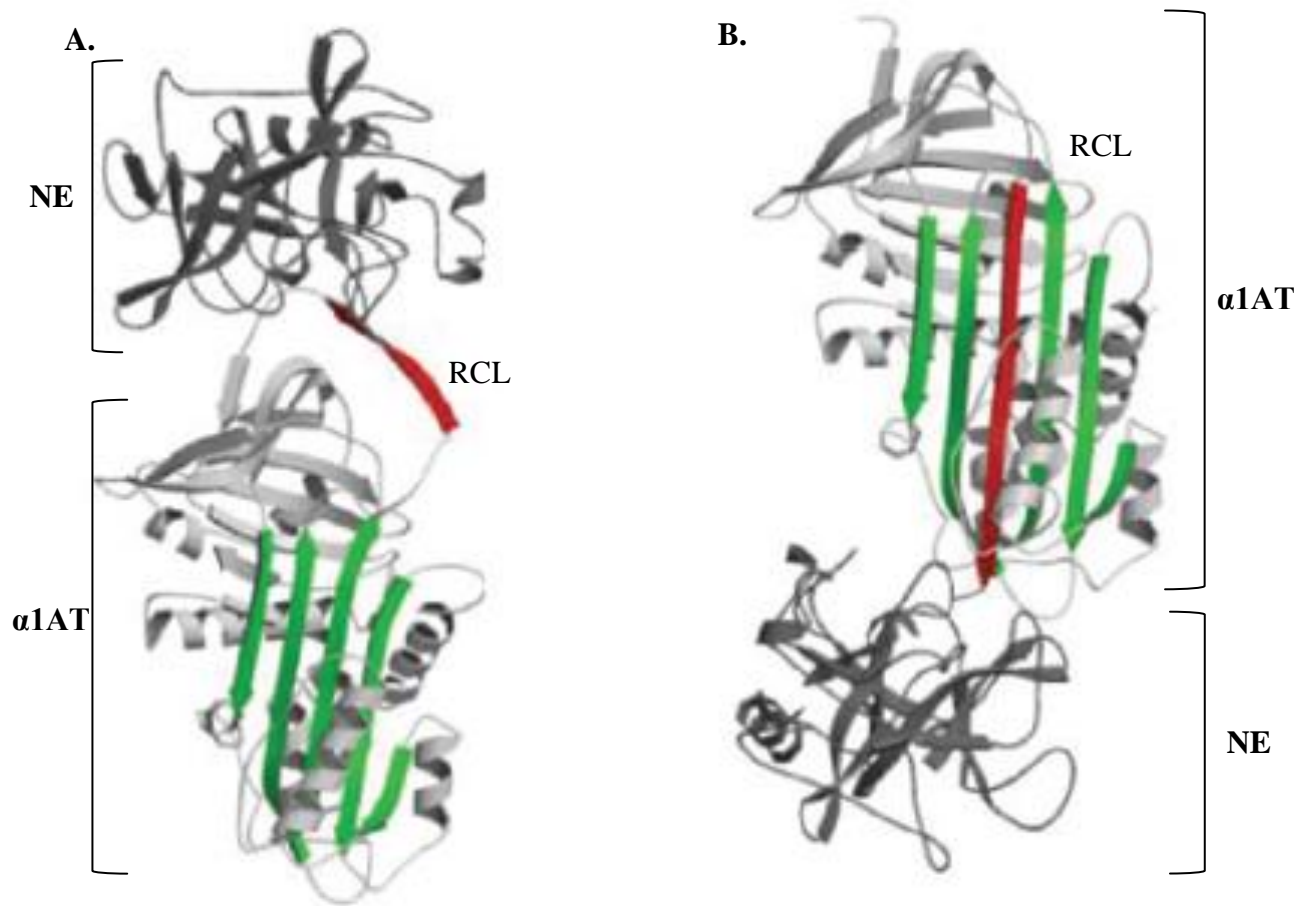


Fig. 4.1.1.7.2. The Protein Structure of α 1AT and its Binding to NE. **A.** The stable, inactive α 1AT with extended reactive centre loop (RCL), depicted in red, protrudes from the main structure attaching to the active region of the NE molecule. **B.** The subsequent movement of the RCL from upper to lower pole position permanently inactivates both proteins. The RCL becomes an additional strand in the beta-sheet A in green (the image above was reproduced with permission from Lomas, *et al*, (Lomas, Belorgey et al. 2005) which was remodelled from an original image by Gooptu, *et al*, (Gooptu et al., 2000) for which there is open permission from the Proceedings of the National Academy of Sciences of the USA.

The main mechanism of NE delivery consists of the release of primary azurophil granules into an internal phagolysosome where the microbe is degraded under controlled conditions. To a lesser degree, exocytosis of the primary azurophil granule into the extracellular spaces can occur (Kessenbrock et al., 2011). However, extracellular NE does not entirely disassociate itself from its originating cell as there is evidence that it is also found on the outer neutrophil surface (Korkmaz et al., 2008b) (Owen et al., 1995) (Allen and Tracy, 1995) where SLPI can bind and inhibit the protease (Zani et al., 2009). While bound to each other, SLPI and NE are thought to be cleared by phagocytic cells *in vivo* (Travis, 1988). SLPI can also help control neutrophil apoptosis and clear the leakage of cellular enzymes (Travis, 1988) thereby dampening the cyclic effect of inflammation. In lung epithelial cells, it has been shown that NE can up-regulate the expression of SLPI (Sallenave et al., 1994) which is able to directly assist with controlling infecting microbes as well as regulate excessive NE. There is strong evidence of the intracellular presence of active SLPI and α 1AT in neutrophils; SLPI has been found in the cytosol and secondary granules in low concentrations (Jacobsen et al., 2008, Sallenave et al., 1997) while α 1AT has been discovered in all neutrophil granules (Clemmensen et al., 2011). This would suggest that neutrophils evolved so that their potentially destructive elastase could be regulated from within their own cytosolic environment. SLPI and α 1AT may play a protective role, on this occasion, within the internal neutrophil environment. A comprehensive list of NE, SLPI and α 1AT's characteristics and functions are summarised in **Table 4.1.1.7.1** and **Table 4.1.1.7.2**.

Details	NE
Protein Accession Number	P08246
Molecular weight, Molecular mass (Protein size)	29,500 g/mol or 29.5 kDa
Amino acids	267 (including signal, propeptide, and chain)
Isoelectric point	10.5 (Korkmaz et al., 2008b, Heutinck et al., 2010) or 10.8 (Travis, 1988), cationic
Glycosylation	Glycosylated (3 sites)
Structure	Single chain glycoprotein
Disulfide bonds	4
Protein family	Serine protease, chymotrypsin family (Korkmaz et al., 2008b)
Main functions	Degrades microbes; damages elastin and ECM components; activation of antimicrobial peptides (LL-37)
Substrate Target	Cleaves after small hydrophobic residues (i.e. valine) (Heutinck et al., 2010). Active site is composed of histidine (residue 70), aspartate (residue 117), and serine (residue 202) which are brought together in a tertiary structure (Korkmaz 2008).
Site for Inhibitors to Bind	Binds to SLPI mainly between the NE amino acid region 214-217 (Koizumi et al., 2008)
Inhibited by	Inhibited by serum α 1-AT in the lungs and vascular circulation (Gadek et al., 1981), SLPI and elafin present in salivary glands (Lee et al., 2002), and α 2-macroglobulin
Cells containing the protein	Mainly neutrophils (Takahashi et al., 1988), monocytes (Campbell et al., 1989), mast cells, eosinophils (Lungarella et al., 1992)
Cells producing mRNA	Bone marrow myelocytic progenitor cells (early stage of granulocyte development) (Takahashi et al., 1988) (Korkmaz et al., 2008b)
Intracellular/ Extracellular	Both intracellular (in azurophil and phagolysosome) and extracellular free or bound
Location in cells	Inside primary azurophilic granules (alongside Cathepsin G and Proteinase 3)
Protein Homology	Cathepsin G (35 %) (Hajjar et al., 2010) Proteinase 3 (56 %) (Hajjar et al., 2010) Azurocidin (Korkmaz et al., 2008b)

Table 4.1.1.7.1. Summary of NE Characteristics

Details	SLPI	α 1AT
Protein Accession Number	P03973	P01009
Molecular weight, Molecular mass (Protein size)	Native form: 11,726 g/mol or 11.7 kDa; <i>In vitro</i> epithelial cell lines, ~10-14 kDa (Sallenave et al., 1997)	51 kDa (Carrell et al.), 52 kDa (Clemmensen et al., 2011), or 55 kDa (Gavrin et al., 2012a)
Amino acids	132	418 (Isoform 1 of a possible 5 being the most common) made up of 394 aa + 24 aa signal peptide (Carrell et al., 1982, Jeppsson and Franzen, 1982)
Isoelectric point	11, cationic, basic	4.4 - 4.7, anionic (Jeppsson and Franzen, 1982)
Glycosylation	Non-glycosylated	N-Glycosylated (3 sites)
Structure	Hydrophobic, acid-stable, single polypeptide chain. Boomerang-like shape, each arm having 1 structurally homologous domain (Domain 1: NH ₂ -terminal or Domain 2: COOH-terminal).	One polypeptide chain with 3 carbohydrate side chains (Carrell et al.) referred to as the β -sheets and exposed reactive centre loop that can relocate
Disulfide bonds	8	None
Protein family	Chelonianin family, Whey acidic protein (WAP)	Serine proteinase inhibitor (Serpin)
Main functions	Inhibits NE and has anti-microbial activity.	Irreversibly inactivates NE
Main target and site of inhibition activity	Inhibits NE by utilizing the SLPI amino acid region 84-132 (C-terminal). Active residue: Leucine position 72 and Methionine 73 (Rudolphus et al., 1991) (Koizumi et al., 2008)	Reactive centre loop at position 358 (Carrell et al., 1982)
Other targets to inhibit	Inhibits cathepsin G, trypsin, tryptase, (pancreas-derived) chymotrypsin, chymase.	Inhibits cathepsin G, proteinase 3, trypsin, chymotrypsin, thrombin (van 't Wout et al., 2012)
Cells containing the protein	Mucosal and oral epithelial cells, secretory acinar cells in salivary glands (Ohlsson et al., 1984), macrophages, neutrophils (Korkmaz et al., 2008b), tracheal & bronchial cells, lung monocytes (Sallenave, 2000), dermal keratinocytes, intestinal goblet-type epithelial cells (Bergenfeldt et al., 1996)	Hepatocytes, neutrophils, monocytes (Mornex et al., 1986), alveolar macrophages (Perlmutter et al., 1985) and freely roaming within plasma/serum
Cells producing mRNA	Same as list above including oral buccal epithelial cells (Nittayananta et al., 2013a), tonsillar mast cells (Westin et al., 1999b), serous nasal glands (Westin et al., 1994), chondrocytes in human articular cartilage (Ohlsson et al., 1997)	Hepatocytes, neutrophils, blood monocytes, alveolar macrophages (Mornex et al., 1986)
Intracellular/ Extracellular	Mainly inhibits in extracellular spaces; but can be found intracellularly	Mainly inhibits in extracellular spaces but can be found intracellularly
Location in cells	In neutrophils, inside secondary granules and the cytosol (Jacobsen et al., 2008)	In neutrophils, inside azurophil, specific, and gelatinase granules as well as secretory vesicles (Clemmensen et al., 2011). In hepatocytes, processed by the endoplasmic reticulum and transported out to serum
Protein Homology	Elafin (42 %)	Ovalbumin (30%), Antithrombin-III (29%) (Carrell et al., 1982)

Table 4.1.1.7.2. Summary of SLPI and α 1AT Details

4.1.1.8. SLPI as a Potential Therapeutic Agent

Recombinant (r)SLPI has been put forth as a potential therapeutic entity due to its proven *in vitro* anti-bacterial (*Escherichia coli* and *Staphylococcus aureus*) (Hiemstra et al., 1996), anti-fungal (*Candida albicans*) (Tomee et al., 1997), and anti-viral (HIV) (McNeely et al., 1995) functions. SLPI regulation of excessive and potentially damaging NE has also made it an important protein to explore for treatment purposes (Tomee et al., 1998). Inhaled administration of rSLPI has been shown to reduce NE and IL-8 levels in the inflamed lungs of cystic fibrosis sufferers (McElvaney et al., 1992) (McElvaney et al., 1993). Aerosolised rSLPI was shown to be stable in epithelial lung fluid of sheep after 6 hrs *in vivo* (Gillissen et al., 1993). If excess NE is shown to cause oral mucosal damage, rSLPI could be considered for use as a preventive or acute treatment for oral ulcers. Its known functions of influencing wound healing (Sumi et al., 2000), its anti-microbial ability, and its NE regulation make this protein an ideal candidate for those who suffer with oral ulcers.

4.1.1.9. α 1AT as a Potential Therapeutic Agent

Current α 1AT therapy centres either on intravenous replacement for individuals suffering with α 1AT-deficiency affiliated COPD (Miravittles, 2012) or inhaled α 1AT protein dissolved in buffer for cystic fibrosis (CF) treatment (Griese et al., 2007). The inhaled α 1AT treatment, Prolastin® (Bayer Corp, USA), significantly reduced the number of neutrophils in CF patients sputum along with pro-inflammatory markers such as IL-8, IL-1 β , and TNF- α (Griese et al., 2007). Again, if excess NE is shown to cause damage to the oral mucosa, there is potential for topical α 1AT administration to prevent or treat oral ulcers. However, a possible disadvantage may be oxidation of the α 1AT by reactive oxygen species generated by oral phagocytic cells and bacteria species thus rendering the protein ineffective (Matheson et al., 1979). An alternative human NE inhibitor, EPI-hNE4, was specifically constructed to resist oxidation due to its lack of methionine residues. It was developed by Diax Corporation (Cambridge, USA) in conjunction with Debiopharm (Switzerland) and was successfully tested *in vitro* using healthy blood neutrophils and CF sputum for anti-NE activity, but has not yet undergone testing *in vivo* (Attucci et al., 2006).

Other low molecular weight NE inhibitors are already available on the market such as Sivelestat (market name Elaspol[®], Ono Pharmaceutical Co., Ltd.) for acute lung damage due to inflammation, but is only available in Japan and South Korea (Lucas et al., 2011). AZD9668 (AstraZeneca, UK), another reversible NE inhibitor drug, has been trialled on those with CF (Elborn et al., 2012) and COPD (Vogelmeier et al., 2012), but failed to show significant improvement when analysing lung function. In Elborn, *et al*'s, clinical trial, the sputum neutrophil counts and elastase activity were no different in cystic fibrosis patients receiving the drug (Elborn et al., 2012), however the IL-6 inflammatory marker was significantly reduced in sputum. Recently in 2013, a small, UK-based Phase II trial found that bronchiectasis patients treated with AZD9668 did show improved lung function, but still had the same numbers of neutrophil in sputum (Stockley et al., 2013). The capacity for these treatments to work systemically in those with vascular inflammation and mouth ulcers has not been explored.

4.1.1.10. SLPI Gene Expression

Previous studies have shown increased expression of human SLPI mRNA in cultured primary bronchial epithelial cells (van Wetering et al., 2000), serous glands in the nasal mucosa (Westin et al., 1994), and saliva glands of healthy versus HIV-infected people (Wahl et al., 1997a) using Northern blot analysis. However, more recently, QPCR has been utilized to measure SLPI mRNA in different cell types. An increased expression was measured in inflamed tissue from human colonic biopsies (Schmid et al., 2007) and in cells harvested from cultured post-mortem human airway submucosal glands stimulated with NE (Saitoh et al., 2001). Normal gingival epithelium and immortalized foetus buccal mucosa have also shown upregulated SLPI mRNA when exposed to purified HIV-1 viral lysate (Jana et al., 2005).

To date, no gene polymorphism or genetic SLPI deficiency associated with a disorder has been described (Vogelmeier et al., 1996). As mentioned previously, a single nucleotide polymorphism (SNP) of the α 1-AT gene has been found to be associated with lung emphysema, liver disease, and anti-proteinase 3 antibody-positive vasculitis (Stoller and Aboussouan, 2005). SLPI has yet to be characterised or measured in BD and RAS. In this study, the sensitive RT-qPCR technique will measure SLPI mRNA from adult human oral buccal mucosa epithelial cells in normal healthy individuals and those with BD and RAS during and in between oral ulceration presentation. This will help to clarify if there are

abnormal SLPI mRNA levels present in BD or RAS patient and whether their epithelial cells respond to an ulcer with an upregulation of SLPI to regulate NE.

4.1.1.11. Inhibitor Inactivation and Other Regulatory Factors to Consider

Just as NE concentrations are controlled, SLPI and α 1AT are also subjected to regulation. SLPI can be cleaved and inactivated by Cathepsins B, L, and S cysteine proteases if they are released from eukaryotic cell lysosomes (Taggart et al., 2001). SLPI is also inactivated by chymase, a serine protease, suggesting an *in vivo* feedback mechanism of regulation (Belkowski et al., 2009). Cathepsin B has been measured in saliva and gingival crevicular fluid (GCF) of persons with periodontitis but was found to be present at a much lower concentration than SLPI or α 1AT (Cox et al., 2006). Cathepsin L has also been measured in saliva (Mirzaii-Dizgah and Riahi, 2011) and can act as a potent elastase (Chapman et al., 1994). Similar to NE and Cathepsin B, Cathepsin L has the potential to degrade various ECM components (Dickinson, 2002). However, Cathepsin B and L are intracellular lysosomal enzymes and therefore have an optimal activity in low pH (3-6.5). If they are released from cells and in a neutral or alkaline pH environment, these enzymes are unstable and permanently deactivated (Dickinson, 2002). This would reduce their digestive capabilities in the oral extracellular environment which is bathed in saliva with a pH normally around 6-7 (Humphrey and Williamson, 2001b) creating an opportunity for released NE to degrade ECM instead. Cathepsin S, another SLPI inhibitor with similar structure to Cathepsin B and L, has been shown to be stable with 25% of its optimal elastase activity at pH 7-8 *in vitro* (Shi et al., 1994, Shi et al., 1992) so could conceivably contribute to both the breakdown of ECM and the inhibition of SLPI. However, the level of Cathepsin S in the oral cavity is unknown as it is mainly expressed by antigen presenting cells such as alveolar macrophages and has a high concentration in the spleen (Shi et al., 1994). Finally, chymase can be found in sputum as it is most likely released from lung and oral mast cell granules. SLPI can inhibit chymase but it has also been shown *in vitro* that chymase can cleave SLPI as if it were its substrate. With all of these potential salivary SLPI inhibitors, it is still not known if the cleavage of SLPI occurs with an unbound or complexed SLPI molecule.

α 1AT can be inactivated by neutrophil-derived collagenase (Michaelis et al., 1990), metalloproteinases (Vissers et al., 1988), and reactive oxygen species (ROS) (Rosenberg et al., 1984) (Vignola et al., 1998) all of which may be present extracellularly in the

mouth. SLPI is also susceptible to inactivation by oxidants (Heinzel-Wieland et al., 1991), heat (Gomez et al., 2009), and cigarette smoke (Cavarra et al., 2001). SLPI's antibacterial activity can withstand an acidic pH of 4.5 (Gomez et al., 2009) but its antiprotease activity is not known at very low pH. α 1AT's ability to inhibit NE is severely reduced at low pH (Schonberg et al., 2012).

α 1AT can inhibit Proteinase 3 while both SLPI and α 1AT can inhibit Cathepsin G. These proteases have similar capabilities of degrading elastin, collagen and proteoglycan as NE but are kinetically slower and therefore considered inefficient. It is for this reason that they have not been directly implicated in tissue damage (Travis, 1988, Reilly and Travis, 1980). Thereby, Proteinase 3 and Cathepsin G were not chosen as part of the primary investigation to the underlying cause and reoccurrence of ulcers, but could still potentially play a minor role.

4.1.2. Hypothesis

The hypothesis of this study is that excessive NE in the mouth is contributing to the cyclic inflammation and the oral ulcerative pathology in both BD and RAS disorders. NE's proteolytic activity in saliva and on the mucosal epithelium may be contributing to oral tissue damage leading to an ulcer. The two main NE inhibitors, SLPI and α 1AT, may be depleted and therefore not able to regulate excessive NE effectively.

4.1.2.1. Objectives:

1. Determine the concentration of NE, SLPI, and α 1AT in saliva from HC compared to BD and RAS patient groups with and without oral ulcers
2. Investigate the activity of NE in saliva and its interaction with various inhibitors
3. Investigate NE, SLPI, and α 1AT protein and complexes in saliva specimens using western blot
4. To identify the location of these proteins in oral mucosal tissue biopsies at the site of ulceration and surrounding the wound
5. Measure the mRNA of SLPI in HC, BD, and RAS patients' oral buccal epithelial cells

4.2. Materials and Methods

4.2.1. Western Blots for Detecting NE, SLPI, and α 1AT Proteins

4.2.1.1. Preparation of Samples and Positive Controls for Gel Electrophoresis

Saliva and isolated OBS protein aliquots were defrosted from the -80°C and kept on ice. The saliva samples had their total protein previously quantified so that the same concentration could be added into each well on the gel. A final volume between 10-25 μl would be added per well including loading and reducing buffers.

Known concentrations of the positive control were run alongside the samples on the same gels. For SLPI assays, the positive control selected was an *E. coli*-derived (Ser26-Ala132) 12 kDa recombinant human SLPI protein (R&D Systems, UK). The NE positive control consisted of the full length 29.5 kDa native, active NE protein isolated from human neutrophils (Abcam, UK). The α 1AT positive control was an in-house preparation of the A549 lung carcinoma epithelial cell line protein extract.

Saliva and OBS samples were prepared in a 1X final volume mixture with NuPage 4X LDS sample buffer (Life Technologies, UK) and NuPage dithiothreitol (DTT)-based 10X Reducing Agent (Life Technologies, UK). This sample mixture was then heated at 70°C for 10 min to reduce the proteins before loading onto the gel. Commercially purchased positive control proteins did not have DTT reducing buffer or heat-treatment as this caused adverse degradation. The gels were NuPAGE[®] Novex 4-12%, 10% or 12% Bis-Tris gels, 1.5 or 1.0 mm thickness, 10 or 12 wells (Life Technologies, UK). At least one lane with the Full Range Rainbow Marker 12-225 kDa (GE Healthcare, UK) was always included on the gels. The gels were run at 70 Volts (V) for 2 hrs or 100 V for 1 hr with the 1X NuPage[®] MES SDS Bis-Tris Running Buffer recommended for small MW proteins. The visible rainbow marker allowed the travelling proteins to be monitored while running the gel.

4.2.1.2. Transference of Proteins to Western Blot Membrane

Fifteen minutes prior to completion of the run, 2 large, shallow plastic dishes were arranged: one containing approximately 10 ml of 100% methanol and the other distilled water (dH_2O). Polyvinylidene difluoride (PVDF) membranes were cut taking great care not to touch the membrane to an average dimension of 9 x 7 cm for the protein transfer from the gel to the membrane. For SLPI, PVDF Immobilon-P^{SQ} membranes with a pore

size of 0.2 μm were used in order to capture small proteins less than 20 kDa while PVDF Immobilon-P membranes with a pore size of 0.45 μm (Millipore, UK) was utilized for NE to capture proteins greater than 20 kDa. Secondary fluorescent antibodies were tested as well, but PVDF Immobilon-FL membrane was used instead in order to avoid a dark background. For all experiments, the PVDF membrane was soaked in 100% methanol for 15 sec to activate and then placed into the dH_2O for 5 min followed by a soak in blotting buffer for 10 min. The blotting buffer, was also the transfer buffer, and was made up of 0.3 % Trizma (Tris) base (Sigma, UK), 1.4 % glycine (Sigma, UK), 20 % methanol (VWR, UK), and dH_2O made up to a 1 L final volume. Subsequently, two white absorbance filter paper supports were also cut to a similar size to that of the membranes and placed in the blotting buffer for 10 min alongside the membranes. The corners of the membranes were handled by clean, flat-tipped tweezers.

When the gel run was complete, the gel cassette was removed and opened with a flat spatula in a shallow bath of blotting buffer. The remnants of the wells were delicately trimmed as well as the bottom part of the gel connecting it to the cassette. A gel-membrane transfer sandwich was assembled from “bottom to top” starting with a plastic cassette (as the backbone), followed by a thick pre-soaked blotting pad, 1 of the pre-cut, pre-soaked filter paper inserts, then the gel and PVDF membrane laid on top without bubbles, and then another filter paper, blotting pad, and top half of the plastic cassette. These layers were clipped together and slotted tightly into a transfer tank filled with blotting buffer and a plastic-enclosed ice block to keep the tank from overheating. The gel was placed on the side closest to the negative cathode as the current passed towards the positive anode carrying with it the negatively-charged proteins across the membrane. Electrophoresis was carried out at 200 mA constant current for 1 hr.

4.2.1.3. Blocking Buffer

When the transfer of proteins was complete, as verified by the rainbow marker and loading dye transferring successfully to the membrane, the cassette was disassembled and the PVDF membrane was removed and placed into a small clean dish containing approximately 10 ml of blocking buffer, enough so that the solution covered the membrane, consisting of 5% non-fat milk prepared from powder (for NE membranes) and 5% goat serum (for SLPI) membranes in 1X PBS with 0.05% Tween-20 (Sigma, UK). Membranes were blocked overnight at 4°C with gentle agitation.

4.2.1.4. Washing Membranes

Blocking buffer was discarded after use and the membrane was washed by gentle agitation for 10 min with 1 X Tris Buffer Solution (TBS) wash buffer consisting of a final concentration of 0.1 M Tris-HCl solution, 0.9% NaCl, 0.1% Tween-20 in dH₂O adjusted to pH 7.5 with hydrochloric acid (HCl). The membrane wash was repeated 3 times.

4.2.1.5. Western Blotting for SLPI

4.2.1.5.1. Primary SLPI Antibody

After extensive optimisation in our laboratory, the most sensitive and specific SLPI primary antibody (1^oAb) that was utilized for the western blot was a goat, anti-human polyclonal (R&D Systems, UK) diluted to 1:500 (equivalent to 1 ng/μl). The primary antibody was diluted in 3% BSA in PBS with 0.05% Tween-20. The membrane was incubated for an hour at room temperature. The membrane was washed 3 times with TBS buffer before addition of secondary antibody.

4.2.1.5.2. Secondary SLPI Antibody

An optimised 1:3000 dilution of the secondary antibody, a horseradish peroxidase (HRP) stabilized donkey, anti-goat IgG conjugate (R&D Systems, UK) was used to detect SLPI. The secondary antibody was prepared in the same diluent buffer as the primary, 3% BSA in 1X PBS with 0.05% Tween-20 and incubated with the membrane for 1 hour at room temperature with gentle agitation. 1X TBS buffer was used to wash the membranes 3 times for 10 min where the wash buffer was changed in between washes.

4.2.1.6. Western Blotting for NE

4.2.1.6.1. Primary NE Antibody

For the NE target, monoclonal mouse IgG1 primary antibody (Clone 265-3K1) which was protein G purified was directly conjugated to a HRP label using Lightening-Link® according to the manufacturer's protocol. (Innova Biosciences, UK). Briefly, in a clean eppendorf tube, 1 μl of the LL-Modifier kit reagent was mixed with every 10 μl of antibody to be labelled, in this case, 10 μl of LL-Modifier to 100 μl of neat 0.1 μg/μl NE antibody. The total amount of antibody in the mix was 10 μg. This solution was then transferred to a vial with the set amount of lyophilized HRP (10 μg) with gentle mixing using a pipette. The final ratio was 1:1 (10 μg HRP: 10 μg Ab). After the antibody and

HRP solution were incubated for a minimum of 3 at RT, 1 μ l of LL-Quencher was added for every 10 μ l of antibody used therefore 10 μ l of Quencher was added in our experiments. After 30 min at RT, the HRP-labelled antibody was ready to be utilised. To store it long term, aliquots were placed in the -80°C for future experiments.

An optimised dilution of 1:500 of NE-HRP antibody was prepared in a diluent solution consisting of 5% goat serum in PBS with 0.05% Tween-20 and incubated for 1 hr at room temperature. Afterwards, TBS wash buffer was used to wash the membranes 3 times for 10 min each session.

4.2.1.7. Western Blotting for Beta-Actin

Even though the samples that were used for the westerns had their total protein quantified, beta(β)-actin (42 kDa) was also used as an additional loading control. Probing for this antigen on the same membrane was conducted after the initial target (SLPI, NE, α 1AT) was developed, detected, and washed.

4.2.1.7.1. Human Beta-Actin Primary Antibody

The rabbit polyclonal β -actin antibody (Abcam, UK) was diluted 1:4000 in 3% BSA in PBS with 0.05% Tween-20 and incubated with the membrane for 1 hr at RT. Afterwards, it was washed thoroughly using the protocol described above.

4.2.1.7.2. Beta-Actin Secondary Antibody

The secondary antibody was a goat, anti-rabbit (Thermo Scientific, UK) diluted at 1:1200. The membrane was gently agitated for 30 min to 1 hr at RT and then washed three times. The protein bands were visualised using electrochemiluminescence (ECL) detection as described below.

4.2.1.8. Development and Detection Using the Dark Room ECL Method

Solutions A and B from the ECL Plus Western Blotting Substrate kit (GE Healthcare, UK) were equilibrated to RT before use. The western blot membrane was drip dried from the corners to remove excess fluid after the last wash and laid across a 20 x 20 cm clean pliable plastic sheet (tear-proof). The sheet was laid out flat to create a surface area on which to incubate the membranes with the developing fluid. Detection solution A and B were combined at a 40:1 ratio into a clean universal vial. The volume added to a 9 x 7 cm membrane differed depending on the protein target (see **Table 4.2.1.8.1**).

Reaction Component	SLPI	NE	β-actin
Detection Solutions and Volume Required	A: 1 ml B: 0.025 ml	A: 1 ml B: 0.025 ml	A: 2 ml B: 0.050 ml
Time Incubated	2 min	3 min	5 min

Table 4.2.1.8.1. ECL Detection Solution and Incubation Time per Membrane. The volume and incubation time for each protein target was optimised to produce the protein bands without a dark background.

Once mixed, the detection solution was pipetted on top of the membrane ensuring that the entire area received full coverage. During the incubation time (detailed in **Table 4.2.1.8.1**), the solution was evenly distributed on the membrane by picking up the sturdy plastic sheet underneath and agitating the liquid. At the end of the incubation period, a corner of the membrane was picked up with flat-tipped tweezers and excess reagent allowed to drain. Next, the membrane was placed on a new, clean plastic sheet which was folded over to enclose the membrane. Air bubbles were gently pressed out from between the plastic sheet and the membrane. The membrane enclosed in the plastic sheet was then fixed with tape to the inside of a development cassette. Inside the dark room a piece of the hyperfilm (Amersham, GE Healthcare, UK) was cut to approximately the same size as the membrane and placed in the cassette on top of the plastic-enclosed membrane. The cassette was closed and left for between 1-5 min for the image to develop. Afterwards, the hyperfilm was placed into the development machine after which the resulting hyperfilm image was laid on top of the plastic protected membrane and aligned with the rainbow ladder in order to mark the exact placement of each kDa band size.

4.2.2. Enzyme-Linked Immunosorbent Assays

4.2.2.1. Recovery Assays for SLPI, NE and α 1AT ELISA's

The validity of each ELISA assay was completed for quality assessment and determination of a recoverable target protein in the presence of human saliva. This was completed by adding a known quantity of purified target protein from the standard provided by each kit such as rSLPI into the specimen being assayed. A starting dilution of

1:200 for saliva was spiked with 800 pg of rSLPI, 5 ng of NE, or 50 ng of α 1AT. These spike concentrations were chosen as they were within mid-range of their ELISA's standard curve. A 1:2 dilution series of the spiked sample was prepared (1:400, 1:800, 1:1600). The saliva sample alone (without any spike) and the purified protein alone were measured separately and their combined concentration was compared to the spiked sample. According to the R&D Systems protocol, acceptable recovery should be within a range of 80-120%. SLPI ELISA's recovery of the spike in saliva was within the acceptable range of 89-99% (average 94.1%) for all four dilutions. The α 1AT ELISA recovery ranged from 70-127% (average 100.96%), the lower recovery being in some of the 1:200 dilutions which means it was perhaps too concentrated. And finally, the NE ELISA which was able to determine an accurate 90-99 % (average 95.5%) recovery in three of the four dilutions, only the 1:1600 being too dilute.

4.2.2.2. The Levels of NE, SLPI, and α 1AT in Saliva: ELISA Assays

Enzyme linked immunosorbent assays (ELISA) were used to measure human SLPI, (Quantikine[®] immunoassay, R&D Systems, UK), human neutrophil elastase (HyCult Biotech kit, The Netherlands), and human alpha1-antiTrypsin (GenWay Biotech, USA). Some of the saliva supernatants were completed on the same day for SLPI and NE assays. Also, a few patients samples were repeated (to check accuracy and stability of the assay's measurements) however newly defrosted saliva aliquots were always used. Samples and standards were run in duplicate. Due to a high amount of protein in saliva, the samples were diluted 1:200 in diluent buffer specific for each kit and 100 μ l added to each well. Blank control wells with the same diluent buffer were included to subtract absorbance background. The number of saliva samples tested in each patient group is summarised in **Table 4.2.2.2.1.**

Patient Group / Mouth Activity	SLPI (n)	NE (n)	α1AT (n)
BDa	52	50	27
BDq	56	57	28
HC	49	55	17
RASa	15	12	3
RASq	11	10	5

Table 4.2.2.2.1. Patient Saliva Sample Size (n) Tested on ELISA

4.2.2.2.1. Human SLPI Immunoassay

SLPI ELISA (Quantikine[®], R&D Systems, UK) was performed as stated in the manufacturer's guide. Briefly, SLPI standard was reconstituted in 5 ml of calibrator diluent which provided a stock of 4000 pg/ml – the highest standard concentration on the plate. A 1:2 series dilution was completed down to 62.5 pg/ml. Calibrator diluent alone was also included. 100 µl of assay diluent was added to each well (which was pre-coated with capture antibody by the company) followed by 100 µl of the diluted saliva samples and standards. The plate was sealed with an adhesive plate cover and incubated undisturbed for 2 hours at room temperature (RT). Each well was then washed with 400 µl of 1X wash buffer (supplied by the kit) using a repeat pipette dispenser. The wash buffer was removed by inverting the plate into a sink. This was repeated another two times and after the last wash, the plate was inverted and blotted against a paper towel to remove excess moisture and any bubbles. 200 µl of SLPI conjugate (polyclonal anti-human antibody conjugated with horseradish peroxidase) was then added to each well, the plate was covered, and incubated for 2 hrs at RT. The same wash procedure was followed. 200 µl of substrate solution (colour reagent A, hydrogen peroxide, and B, the chromogen tetramethylbenzidine (TMB) previously combined in equal volumes) was added to each well and the plate was incubated in the dark for 20 min. 50 µl of 2N sulfuric acid stop solution (2N H₂SO₄) was then added to each well and the absorbance was read within 30 min at 450 nm with a secondary reading at 570 nm for wavelength correction using the OPTIMA Microplate Reader (BMG LabTech, Germany). The 570 nm was subtracted from the 450 nm reading. Absorbance results from the samples and rSLPI standards were correlated to create a standard curve in order to interpret the sample protein concentrations.

4.2.2.2.2. Human Elastase Kit

Saliva samples were thawed on ice before being diluted 1:200 using the NE ELISA (HyCult Biotech, The Netherlands) kit's diluent buffer. The NE standard was reconstituted with dH₂O as specified by the manufacturer. A 1:2 standard dilution series was prepared for a final concentration range between 0.4 – 25 ng/ml. 100 µl of both standards and samples were added to the wells (which were pre-coated with capture antibody) in duplicate on the 96-well plate provided. Blank wells were also included with dilution buffer alone to subtract background absorbance. An adhesive cover was applied

to the plate and the plate was incubated for 1 hr at RT. After which the plate was inverted over a sink and the liquid decanted. Each well was washed 4 times with 200 μ l 1X wash buffer. The plate was inverted again and blotted on paper towels and 100 μ l of diluted tracer was added to each well, an adhesive cover applied to the plate, and incubated for 1 hr at room temperature. The same wash procedure was completed. 100 μ l of streptavidin-peroxidase was added to each well. The plate was covered, and incubated for 1 hr at room temperature then washed again 4 times. 100 μ l of TMB (Sigma, UK) was then added to each well. The plate was covered and incubated for 20 min in the dark at room temperature after which 100 μ l of stop solution 2N H₂SO₄ was added. The plate was read within 30 min at 450 nm absorbance. All NE concentrations were determined from a standard curve generated by the absorbance of the dilution series. Background was subtracted from the standards and sample values.

4.2.2.2.3. Human Alpha1-AntiTrypsin

The capture antibody provided in the kit (GenWay Biotech Inc., San Diego, CA, USA) was diluted 1:1000 in 0.05 M carbonate-bicarbonate solution (pH 9.6) and 100 μ l was added to each 96 well of a NUNC absorbent plate. After incubating overnight at 4°C, fluid was discarded and each well was washed three times by adding 300 μ l wash solution (0.05% Tween 20 in PBS, pH 7.4) and decanting. 200 μ l Blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8) was then added and the plate was incubated for 1 hour at RT followed by another wash step. Saliva samples were defrosted and diluted 1:200 in diluent buffer consisting of same constituents as the blocking solution but with 0.05% Tween-20. (Serum samples were diluted 1:100,000 since α 1AT has been reported as having a high concentration in serum (Mornex et al., 1986)). Standards were also prepared in a 1:2 dilution series from 90 – 1.4 ng/ml. Duplicate wells each with 100 μ l of diluted sample, standards, or diluent buffer alone were added to the appropriate wells and incubated for 1 hr at RT. Wells were then washed 5 times. Detection antibody HRP was diluted 1:2500 and 100 μ l was added to each well, incubated for 1 hr at RT, followed by 5 washes. To each well, 100 μ l TMB enzyme substrate was added and the plate kept in the dark for about 15 min. To stop the reaction, 100 μ l of 2M H₂SO₄ was pipetted to each well. Subsequently, the absorbance values at 450 nm were read on the OPTIMA plate reader (BMG LabTech, Germany). All α 1AT concentrations were determined from a

standard curve generated by the absorbance of the dilution series. Background was subtracted from the standards and sample values.

4.2.2.2.4. Statistical Test for ELISA's, Enzyme Activity, and qPCR Assays

The median concentrations in each group were compared to one another using the Mann Whitney U, non-parametric (2 tailed) statistical test for two independent samples. A non-parametric test was chosen for several reasons. 1) Most of the data was skewed and there were some outliers. To try to correct this, the data was transformed, but not all the patient groups could achieve a normal distribution curve. This was confirmed using the Shapiro-Wilk test of normality. 2) Although decent sample sizes were collected from HC and BD patients for ELISA, not enough RAS patients could be recruited for research participation. Their sample number was < 20.

4.2.3. Protein Isolation from Oral Buccal Swabs

4.2.3.1. Initial Protein Isolation Protocol

The initial kit that was used for the isolation of protein was the Illustra Triple Prep kit (GE Healthcare, UK) which isolated DNA, RNA, and protein from the same sample however this kit was eventually discontinued as it did not yield enough RNA for qPCR. However, the protein that was purified was acceptable. A lysed OBS sample was centrifuged through a DNA column and the flow-through containing the protein collected. It was then transferred to a new 1.5 ml microcentrifuge tube and 600 µl of protein precipitation buffer type 1 was added. After vortexing, the sample was incubated for 5 min at room temperature in order to precipitate the proteins then centrifuged for 10 min at 16,000 x g. Supernatant was removed by pipette and the protein pellet was washed in 1 ml of dH₂O to disperse the pellet. The sample was centrifuged at 16,000 x g for 1 min and supernatant was removed carefully using a pipette. The OBS protein pellet was then suspended in 100 µl of 2-D DIGE Buffer which consisted of a final concentration of 7 M urea (Sigma), 2 M thiourea (Sigma), 30 mM Tris-HCl (Sigma), and 4% CHAPS (Sigma) dissolved in dH₂O. The final pH of the 2-D DIGE Buffer was adjusted to 8.5 with 1 M NaOH. Aliquots of the protein were stored in the -80°C until used for western blots.

4.2.3.1.1. Alternative Protein Isolation Protocol

An alternative dual RNA and protein isolation kit, the RNeasy Micro Kit (Qiagen, UK), provided an improved, high quality and quantity RNA yield than the Illustra Triple Prep

kit and so was used for the remainder of the study. After the lysed sample passed through the kit's MinElute column for the first time, the 700 µl of flow-through containing the protein was placed on ice and processed after the RNA was isolated (see **Section 2.1.7.1**). This was transferred to a 15 ml Falcon tube and 4 volumes, or 2.8 ml, of 100% ice cold acetone was added. After a gentle mixing by inversion, the sample was incubated in the acetone for 30 min on ice. It was then centrifuged at 3500 x g for 10 min. The supernatant was discarded and the protein pellet left to air dry. The buccal scrape protein pellet was resuspended in 100 µl of 2-D DIGE Buffer as before and kept at -80°C until use on a western blot.

4.2.4. Measuring Total Protein Concentration in Saliva and OBS Samples

Protein concentration was determined using the 2D-Quant Kit (GE Healthcare, UK). The kit included a protein precipitant, co-precipitant, working colour reagent, and copper solution. The kit provided 2 µg/µl of BSA to use as a standard with a suggested range of 0 – 50 µg per reaction. Duplicate standard tubes were prepared with a selected quantity of BSA for 7 standard points (i.e. 2.5 µl of 2 µg/µl of BSA would give 5 µg, etc, up to 50 µg). Patient samples were defrosted and 5 µl was aliquoted into a new 0.5 ml labelled tube. Then, 67 µl of precipitant was added to each tube of standards and samples, tubes were vortexed briefly to mix, and incubated for 3 min at RT. An equal volume of co-precipitant, 67 µl, was added to each tube and vortexed. All samples were centrifuged at 16,000 x g for 5 min to sediment the protein. The supernatant was removed carefully using a pipette and discarded. The tubes were pulse spun again and any remaining liquid was pipetted and discarded with care to avoid disturbing the protein pellet.

The protein was then dissolved in 13.3 µl of copper solution and 54 µl of dH₂O and vortexed to mix. The Working Colour Reagent was prepared by adding 100 parts of A to 1 part of B. 134 µl of the working colour reagent was added to the suspended protein and mixed immediately by inverting the tubes several times. After transferring each 200 µl reaction to transparent 96 well plate and incubation time of 20 min, the absorbance at 480 nm was read on the OPTIMA Microplate spectrophotometer (BMG LabTech, Germany). The data was analysed as an inverted assay where the absorbance values decrease with increasing of protein concentration. The protein concentrations of the samples were determined based on the absorbance of the standards.

4.2.5. Protease Enzyme Activity and Inhibition Assays

4.2.5.1. Principle of the EnzChek® Assay

The basic kinetics of the EnzChek® Elastase Assay Kit (Molecular Probes/Life Technologies, UK) is that a sample containing protease (such as elastase) will digest the elastin substrate which has been conjugated to a quenched BODIPY® FL dye (DQ™ elastin) thereby releasing fluorescence. If a protease inhibitor is added, the digestion of the DQ™ elastin by the protease in the sample should be reduced or inhibited thereby reducing the detectable fluorescence. However, if there is not enough inhibitor to reduce the protease activity, then some fluorescence may be released. Saliva samples were tested using this kit to measure the total protease activity as well as the specific neutrophil elastase enzymatic activity.

4.2.5.2. Protease Activity Assays

4.2.5.2.1. Total Protease Activity in Saliva Samples

The protease activity in saliva was compared in HC (n=30), BDq (n=33), and BDa (n=29). The EnzChek® Elastase Assay kit's reagents were reconstituted as described in the manufacturer's guidelines. Aliquoted stock concentrations of the N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone inhibitor (10 mg/ml stock, equal to 19.88 mM) and porcine elastase (P.Ela) (100 U/ml) were kept in the -20°C until use. 50 µl of each neat saliva sample (for a 1:4 final reaction dilution) was mixed with 100 µl of 1X reaction buffer (which was made up of dH₂O, 0.1M Tris-HCl, 0.2mM sodium azide) and 50 µl of 100 µg/ml DQ™ Elastin. An active elastase (positive) control reaction incorporated 100 µl of 0.2 U/ml of P.Ela to give 0.1 U/ml (or 0.02 units per reaction (U/rxn)) in a final volume of 200 µl. 50 µl of 100 µg/ml DQ™ Elastin and 50 µl of 1X reaction buffer were also added to the control reaction. Negative control wells with 50 µl 1X reaction buffer and 50 µl of 100 µg/ml DQ™ elastin were included in order to deduct background fluorescence from the samples and controls. All reactions were tested in duplicate. The sample plate was placed immediately into the spectrophotometer after DQ™ elastin was added.

Initial fluorescence values were read by the OPTIMA FLUOStar Microplate spectrophotometer (BMG LabTech, Germany) at an excitation wavelength of 485 ± 10 nm and an emission wavelength of 530 ± 15 nm. (Our microplate was able to excite at

480 nm and emit 520-P, where P signifies a wide band width). Gain fluorescence was adjusted for all reactions on the plate in order to normalise to the highest fluorescence for a baseline reading. The ‘intensity required’ value was set between 10-50% as a large fluorescent increase was expected. Fluorescence measurements were acquired every 5 min for a total of 95 min.

Analysis of the fluorescence results was completed using Microsoft Excel software. The saliva samples (which had previously had their total protein content quantified) were normalised to a total protein content of 1.5 µg/µl and the fluorescence adjusted according to the formula:

$$\frac{\text{difference in fluorescence from time zero to end of assay (nm)} \times \text{desired total protein quantity to normalise all samples to (}\mu\text{g}/\mu\text{l)}}{\text{Total protein quantification of sample (}\mu\text{g}/\mu\text{l)}}$$

A large change in fluorescence from time point zero to 95 min indicated high protease activity.

4.2.5.3. Inhibition Assays

4.2.5.3.1. The Inhibition of Purified NE by rSLPI (Proof of Principle)

The ability of recombinant SLPI protein (rSLPI) (R&D Systems, UK) to inhibit active NE (Abcam, UK) was tested by combining different concentrations of each in a fluorescence assay. The N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone inhibitor, P.Ela, rSLPI, and/or NE were added to designated wells of a black 96 well NUNC® plate from the working stocks described below. All reaction wells had a final reaction volume of 200 µl and were completed in duplicate.

In separate reaction wells, 1.08 µM or 2.14 µM of rSLPI was combined with 0.1, 0.05, or 0.045 U/rxn of NE. Two separate reactions were set up with the 1.08 µM and 2.14 µM rSLPI concentrations mixed with 0.01 U/rxn of P.Ela. A combination of 0.045 U/rxn NE and 75 µM of N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone inhibitor, which is a specific inhibitor of elastase, was also used as a control reaction to confirm inhibition of NE activity. A complete inhibition control (referred to as the negative control) was also

tested combining a known inhibitory concentration of 75 μM of the same ketone inhibitor with 0.01 U/rxn of P. Ela.

Just before measurements were taken by the spectrophotometer, 50 μl of 100 $\mu\text{g/ml}$ DQTM Elastin with BODIPY[®] FL conjugate (the fluorescence substrate) was pipette mixed into each well for a final reaction concentration of 25 $\mu\text{g/ml}$. Blank wells with only 1X reaction buffer and 50 μl of 100 $\mu\text{g/ml}$ DQTM elastin were included in order to deduct background fluorescence from the samples and controls. The gain adjustment and fluorescence readings were conducted in the same manner as described in the previous experiment.

4.2.5.3.2. Testing the Inhibition of Endogenous NE in BDa Saliva Using Various Concentrations of Chloromethyl Ketone

A -80°C frozen saliva sample from a BDa patient was defrosted on ice and diluted 1:4 in 1X reaction buffer from the EnzChek[®] Elastase Assay kit. N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone was tested at different final concentrations of 25 μM , 50 μM , 75 μM , 100 μM , 150 μM coupled with 100 μl of the diluted saliva sample to a final saliva dilution of 1:8. As an active elastase control, purified neutrophil elastase was added to 50 μl of 100 $\mu\text{g/ml}$ DQTM Elastin substrate. As an inhibition control, 50 μl of 100 μM ketone inhibitor stock was combined with 100 μl of 0.2 U/ml P.Ela. 50 μl of 100 $\mu\text{g/ml}$ DQTM Elastin was added to all reactions just before being read on the spectrophotometer. Any difference in volume was made up to 200 μl with 1X reaction buffer. All reactions were tested in duplicate. The gain adjustment and fluorescence readings were conducted in the same manner as described in the previous experiment.

4.2.5.3.3. Measuring Specific Neutrophil Elastase Activity in Saliva

BDa n=7, BDq n=6, RASa n=5, RASq n=5, and HC n=8 saliva samples were selected for the measurement of specific NE enzymatic activity using the EnzChek[®] Elastase Assay kit. Using two aliquots of the same sample, the total protease activity (without inhibitor) and the total protease activity with specific neutrophil elastase inhibitor was measured. BDq, RASq, and HC saliva samples were diluted to 1:20 in the final reaction. BDa and RASa were diluted to 1:40 in the final reaction because the concentration of NE was

predominantly higher in these samples compared to saliva from non-ulcer patients and would saturate the elastin too quickly.

100 μ l of each diluted saliva sample was added to 50 μ l of 1X reaction buffer while in a different well the same volume of saliva was added to 50 μ l of 800 μ M of specific NE inhibitor, *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, giving a final inhibitor concentration of 200 μ M. 50 μ l of 100 μ g/ml DQTM Elastin was added to each reaction. The active elastase control consisted of 100 μ l of 0.2 U/ml of P.Ela., 50 μ l of 1X reaction buffer, and 50 μ l of 100 μ g/ml DQTM Elastin. The inhibitor control was prepared as well with 100 μ l of 0.2 U/ml P.Ela, 50 μ l of 100 μ M Inhibitor, and 50 μ l of 100 μ g/ml DQTM Elastin. Blank wells with only 1X reaction buffer and 50 μ l of 100 μ g/ml DQTM elastin were included in order to deduct background fluorescence from the samples and controls. All reactions were tested in duplicate. After an initial gain adjustment, fluorescent readings were detected on the spectrophotometer every 5 min for a total of 95 min run at 37°C. An increase in fluorescence during 95 minutes indicated high protease activity in the sample. The saliva samples that were diluted 1:40 had their fluorescence values multiplied by 2 in order to directly compare the values from the 1:20 saliva samples. The difference between neutrophil-specific elastase activity and total protease activity (i.e. with and without inhibitor) was calculated.

4.2.6. QPCR for OBS SLPI and Reference Gene

4.2.6.1. Primer Design for Selected Reference Genes and Target mRNA

Messenger RNA (mRNA) sequence from the Nucleotide PubMed database was copied and uploaded into the Primer3 and Roche primer design programs. From here suggested primers were individually adjusted using the oligonucleotide properties calculator (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) and the Sigma Design Centre (<http://www.sigmaaldrich.com/configurator/servlet/DesignCenter>) to help check primers for potential hairpins, secondary structures, and primer dimer. Coupled forward and reverse primers were also checked for melting temperature (T_m) compatibility so that both would anneal to their target site at the same temperature. The primer sequence was uploaded into the Basic Local Alignment Search Tool (BLAST) in order to check for organism, gene, and site specificity. An ideal design for mRNA (cDNA) target would be an intron-spanning primer; however, since all RNA samples were treated with a second genomic DNase digestion using the TURBO DNA-freeTM (Ambion,/Life Technologies, UK), this type of primer design was not crucial.

All primers were designed based on human mRNA database sequences taking into account any multiple transcript variations (**Table 4.2.6.1.1**). PPIA, PGK1, and GAPDH sequences were selected from the RTPrimerDB public database after running a reference gene panel (see **Chapter 6**). Using mFold software, the predictive secondary structure of the target at a 64°C annealing temperature was mapped in order to see where the primer would bind and if the area is easily accessible.

Primer Name	Primer Design Sequence	Primer T _m	Product Size
SLPI_F	5' CTGTGGAAGGCTCTGGAAAG	63.9 °C	66 bp
SLPI_R	5' GGCACCTGGGCAGATTTCTTA	64.0 °C	
PPIA_F	5' TCATCTGCACTGCCAAGACTG	66.6 °C	71 bp
PPIA_R	5' CATGCCTTCTTTCACTTTGCC	65.7 °C	
PGK1_F	5' ATGGATGAGGTGGTGAAAGC	63.9 °C	118 bp
PGK1_R	5' CAGTGCTCACATGGCTGACT	64.3 °C	
GAPDH_F	5' AGCCACATCGCTCAGACAC	64.3 °C	66 bp
GAPDH_R	5' GCCCAATACGACCAAATCC	64.0 °C	

Table 4.2.6.1.1. Primers Selected for OBS Sample Gene Analysis. DNA oligonucleotides primers were synthesised by Sigma, UK. F, forward primer. R, reverse primer. Melting temperature (T_m); refers to the degree in Celsius (°C) at which the primer will anneal to the DNA template. The expected product size is described in base pairs (bp).

4.2.6.2. Initial Primer Testing

The primers underwent an initial reaction with Sybr Green mastermix (Roche, UK) and at least 2 different cDNA OBS samples to check that they were amplifying one product. In order to assess this, the T_m of the amplicon was obtained and water (negative) controls were monitored for any amplification. Product sizes were double checked by electrophoresis on a 1% agarose gel.

4.2.6.3. Producing cDNA for Use in Standard Curves: Purification of cDNA Standard

After producing the correct DNA amplicon from the primer test, this amplicon or DNA product was purified from all the other PCR components (such as the enzymes and dNTPs) in order to be used for preparation of standard curves for absolute quantification.

Using the QIAquick PCR Purification kit (Qiagen, UK), the DNA product was collected and mixed with 5 times the amount of PB Buffer, which is a chaotropic salt reagent included in the kit. This mixture was pipetted on top of the provided spin column with collection tube and centrifuged at 13,000 rpm for 1 min at room temperature. This bound the DNA to the silica column. The flow through was discarded in a specialised hazardous waste container for chaotropic salts. Next, 0.75 ml of ethanol-based PE buffer was added to the column and centrifuged again at 13,000 rpm. The flow through was discarded and the column re-centrifuged to pool any residual ethanol. The collection tube was then discarded and replaced with a 1.5 ml microcentrifuge tube. This was followed by addition to the top of the column of 30 µl of either DNase free water or elution buffer provided by the kit was added directly to the top of the column. The column was incubated for 1 min, and centrifuged again for 1 min at 13,000 rpm. The solution eluted from the column contained the purified DNA. The quantity and quality of the DNA was assessed using a NanodropTM spectrophotometer. Good quality DNA gave a value of between 1.7 – 2.0 from the 260/280 ratio.

In order to determine dsDNA copy number of the purified amplicon, the formula given below was completed which takes into account the length in base pairs (bp) and quantity of the DNA. First, the molecular weight (MW) was determined by multiplying the number of bp in the amplicon by 665 (which is the assumed average weight of a base pair). The quantity of DNA (ng) was measured in 1 µl of sample using the Nanodrop and was incorporated into the formula below in order to determine DNA copy number.

Formula:

$$((\text{ng/MW}) / 1 \times 10^9) \times 6.02 \times 10^{23} \text{ molecules/mole} = \text{number of copies (per } \mu\text{l)}$$

The procedures involving amplified products were performed in an area of the lab designated for Post-PCR handling. A separate lab bench, pipettes, filter tips, racks, tubes, coat, gloves, vortex and centrifuge were all part of the 'clean set up' to minimise risk of PCR contamination. An ultra violet (UV) hood was utilised for qPCR mastermix set up as well.

4.2.6.4. Optimisation of qPCR assay

The assay efficiency goal is 100% or as referred to by the Roche Light-Cycler Software as "2.0". This is determined by the slope of the amplification curves where Efficiency = $(10^{-1/\text{slope}})-1$. A slope of -3.32 would equal 100% amplification efficiency (Bustin, 2004). Therefore, considering this in terms of cycles, the best amplification efficiency would be generated from intervals of 3.3 quantification cycles (Cq) for a 1/10 dilution series standard curve.

Since a pre-mixed 2X Sybr Green I Mastermix (Roche, UK) was used for all reactions, and therefore dNTPs, Taq polymerase, and MgCl₂ concentrations were all pre-determined by the manufacturer, special consideration was spent on forward and reverse primer concentration as well as annealing temperature and time in order to optimise the individual assays.

4.2.7. Immunohistochemistry of Oral Biopsies

4.2.7.1. Sectioning Frozen Oral Biopsies

Frozen biopsies were relocated to a cryostat (Bright Instruments Co Ltd, UK) to acclimatise to -20°C from -80°C. The base on the biopsy which sat on a small piece of cork was mounted onto a round metal specimen chuck with a small drop of OCT (Tissue-Tek®, Sakura® Finetek, VWR, UK) medium. The sections were cut with Magnacut disposable microtome blades (Bright Instruments Co Ltd, UK) at 5 - 8 µm and collected onto Polysine® or electrostatically charged Superfrost® Plus glass microscope slides (Menzel-Gläser, Thermo Scientific). Each slide was labelled with the patient's ID and each section was numbered consecutively. The orientation was checked after the first few sections with both Giemsa stain and hematoxylin and eosin (H&E) stain to make sure that all layers of the tissue could be seen in one section (i.e. the mucosal epithelium, lamina propria, and submucosa) These stains also detailed various cells and tissue components.

4.2.7.2. Giemsa or Hematoxylin and Eosin (H&E) Stain for Orientation

4.2.7.2.1. Giemsa Staining

Prior to immunocytochemical staining, the biopsies were stained with Modified Giemsa stain (Sigma,UK) or H&E stain in order to confirm that the biopsy tissue was being cut so that epithelial and submucosa could be seen in one section. For the Giemsa protocol, the biopsy on the slide was fixed in 70% ethanol for 10 min. It was briefly dried and a drop of the concentrated Giemsa solution was added on top of the section and incubated for only 30 sec. Afterwards, it was gently rinsed twice in ddH₂O and allowed to air dry (but not for more than 5-8 min). A drop of DPX mounting media (Sigma, UK) was added on top of the section and a coverslip applied immediately. After the mounting reagent had dried, the biopsies were able to be imaged using a light microscope (Nikon Instruments, UK).

4.2.7.2.2. H&E Staining

The cellular detail of tissue biopsies were revealed using haematoxylin with eosin counter stain. Briefly, samples were rinsed with sterile distilled water before incubation with alum haematoxylin for 5 mins at room temperature to distinguish nuclei, followed by two seconds exposure to 0.3% acid alcohol to enhance contrast. The tissue was rinsed once more and counter stained with eosin for two minutes at room temperature to reveal additional tissue structures and finally the samples were allowed to dry before mounting onto cover slips. Imaging was conducted on a Zeiss Axioskop light microscope (Zeiss, UK).

4.2.7.3. Fixing, Blocking, and Immunofluorescent Antibody Staining of Oral Biopsies

The sections were briefly dried, a Dako Cytomation hydrophobic ink pen (DAKO, Denmark) was used to encircle each specimen on the slide, and 150 µl of 100% ice cold methanol was pipetted on top of the section for 10 min to fix the tissue. Each section was washed with 150 µl of 1X PBS with 0.05% Tween-20 with changing of the wash buffer every 5 min for a total of 15 min. The same blocking buffer as utilised for the immunocytochemical staining of neutrophils (5% goat serum, 0.5% saponin, and 0.05% Tween-20 in PBS) was added and incubated for 1 hr at RT and then removed. The

primary antibody was diluted according to **Table 4.2.7.3.1** and 100 µl was added to the top of the sections which were left undisturbed overnight at 4°C. Isotype and secondary antibody-only controls were also prepared on separate sections.

Target	Block and Permeabilise	Primary and Secondary Antibodies	Antibody Dilution	Isotype Control
mNE	5% goat serum 0.5% saponin 0.05% Tw-20 in PBS	1° Ab: mouse monoclonal IgG1, 0.1 mg/ml neat concentration, (HyCult #HM2174) 2° Ab: AF555 goat, anti-mouse IgG polyclonal F(ab') ₂ region, 2mg/ml (Life Technologies, UK, Cat #A-21425) (visible in red channel)	1: 400 1:2000	Mouse IgG1 (DAKO)
pNE	5% goat serum 0.5% saponin 0.05% Tw-20 in PBS	1° Ab: NE polyclonal rabbit, anti-human, whole antiserum, IgG (Abcam, UK, Cat# ab21595) 2° Ab: AF647 goat, anti-rabbit IgG polyclonal F(ab') ₂ fragment, 2mg/ml (Life Technologies, UK) (visible in far red channel)	1: 400 1:2000	Normal rabbit serum
SLPI	5% goat serum, 1% BSA, 0.5% saponin, 0.05% Tw-20 in PBS	1° Ab: monoclonal mouse IgG1, anti-human (R&D Systems, Cat# MAB1274) 2° Ab: AF555 goat, anti-mouse IgG polyclonal F(ab') ₂ region, 2mg/ml (Life Technologies, UK) (visible in red channel)	1:100 1:2000	Mouse IgG1 (DAKO)
DNA	n/a	DAPI: (Sigma, UK) (visible in blue/UV channel)	Final conc: 100 ng/ml	n/a

Table 4.2.7.3.1. Antibody Details for Detecting NE and SLPI in Oral Mucosal Biopsies. The primary and secondary antibodies used for detecting NE and SLPI in oral ulcer biopsies are listed above. Monoclonal (m) or polyclonal (p) NE primary antibodies were used interchangeably depending on the coupling of another primary antibody.

The next day, the biopsies were rinsed and washed with 150 µl of PBS-T three times for 5 min at a time. The secondary antibody was diluted as indicated in **Table 4.2.7.3.1.** and 100 µl added to the sections. Sections were incubated was for 1 hr at RT while being protected from light. Another triple wash step was then completed. Finally, a 100 ng/ml solution of DAPI stain was prepared in 1X PBS. 100 µl of this solution was added to each section and incubated for at least 5 min before being removed. By placing the slides vertically for a minute allowed the left over fluid around the biopsy section to dry off.

Moving the slide so that it lay flat again, approximately 6 µl of mowial was pipetted on top of the section (mowial was pre-prepared; see section **2.1.8**) and a small 13mm glass coverslip placed on top ensuring there were no air bubbles between the coverslip and specimen. This was covered with aluminum and allowed to dry for at least 1-2 hrs. A thin layer of nail polish was used to seal the sides of the coverslip as to protect the tissue specimen and mounting media from drying out. Slides were stored at 4°C until imaged on a Leica fluorescent microscope using MetaMorph software.

4.3. Results

4.3.1. Western blots: Identification of NE, SLPI, and α1AT in Oral Specimens

Saliva samples were run on Western Blots (WB) in order to see if NE, SLPI, and α1AT proteins could be detected in the specimens. Re-suspended saliva cell pellets and purified total protein from oral buccal swabs were also subjected to this detection process. Recombinant SLPI and purified NE were run alongside these specimens as positive controls for the correct size of the proteins and confirmation of antibody detection.

4.3.1.1. Optimisation of Antibodies for Western Blot

The optimisation of the electrophoresis, protein transfer, and western blot (WB) completed for NE and SLPI targets mainly consisted of variations in incubation times, blocking reagent, antibody types and concentrations meanwhile remaining consistent with the use of the same running and transfer buffers, Bis-Tris gels, PVDF-type membranes, and equipment.

4.3.1.1.1. Issues with Non-Specific Binding of Secondary Antibodies in Oral Specimens

While investigating NE and SLPI saliva proteins on western blots, there were problems with the specificity of several secondary HRP and fluorescent antibodies from various manufacturers (**Table 4.3.1.1.1**). When incubating the membrane without primary antibody, there was a reoccurring 31 kDa protein band consistently present (data not shown). This revealed that there was non-specific binding of the secondary antibodies to an unknown element in all samples including saliva, oral buccal protein, saliva cell pellets, and even human serum. In order to try to block this non-specific binding, 5% goat

serum was used in place of 5% milk or BSA, but this did not alter the outcome. It was clear that the non-specific binding was due to something in the clinical samples because there was no non-specific binding associated with the recombinant SLPI protein or purified NE both of which were isolated by their manufactures. Saliva has over 1,000 proteins and is extremely heterogeneous (Hu et al., 2005) so it was not surprising that there were some conflicts with antibody specificity. These control experiments were paramount in assessing the specificity of the antibodies involved. After many trials, two successful secondary antibodies were found to have specific binding. These are listed in **Table 4.3.1.1.1.1.**

WB Secondary Antibody Details	Supplier or Manufacturer	Dilution Tested	Specific Binding?
Secondary Antibody (Ab), Goat, Anti-Mouse, IgG, HRP, Neat Conc: 10 µg/ml	Thermo Scientific Cat# 32430	1:1000 & 1:4000	No
Secondary Ab, Goat, anti-mouse, HRP	Millipore	1:2500	No
Secondary Ab , Rabbit, anti-mouse, IgG, HRP	Abcam	1:2500	No
Secondary Ab, Polyclonal IgG, Goat, anti-Rat, HRP affinity purified chromatography	Millipore/ Chemicon Intl.	1:5000	No
Fluorescent polyclonal, (red IRDye680LT), Secondary Ab, Goat, Anti-Rabbit, Neat Conc: 1 mg/ml	Licor Cat# 926-68021D	1:15,000	No
Fluorescent polyclonal, (green IRDye800CW), Secondary Ab, Goat, anti-mouse, Neat Conc: 1 mg/ml	Licor Cat# 926-32210	1:15,000	No
Secondary Ab, Goat, anti-Rabbit, HRP, Neat Conc: 10 µg/ml	Thermo Scientific Cat#32460	1:1200	Yes
Secondary Ab, polyclonal Donkey, anti-goat , Goat IgG, HRP	R&D Systems Cat# HAF109	1:3000	Yes

Table 4.3.1.1.1.1. Secondary Antibodies Showing Specific and Non-Specific Binding in Human Saliva Samples. Eight secondary antibodies were tested for target protein specificity on a western blot with saliva samples by replacing the primary antibody with buffer followed by incubating the membrane with the secondary antibody. Six out of the eight secondary antibodies attached to an unknown 31 kDa protein and therefore could not be used. Only two secondary antibodies were found to be suitable for use with saliva proteins. The item catalogue (Cat) number (#) is given where available. **Ab**, Antibody. **Conc**, Concentration. **HRP**, horseradish peroxidase. **IgG**, immunoglobulin G. **WB**, Western blot.

Due to its consistency, the 31 kDa unknown protein causing the non-specific binding was investigated further. A BDq patient saliva sample was run on a western Bis-Tris gel, stained with 0.1% Coomassie Brilliant Blue, destained in an acetic acid/methanol solution, rinsed with water, excised and sent for N-terminal protein sequencing at AltaBiosciences, UK. The results were only 50% accurate inclusive of residues 1-8 (data not shown). The sequence's initial outcome was D-I-Q-L-T-Q-S-P which most closely matches human Ig kappa chain V-I region BAN. Alternative sequences with the queried residues were also researched on protein databases but with limited matches. The reasons for sequencing the protein at this location was important since NE appears around the same size (29.5 kDa) and the specificity of detecting NE in the samples had to be clarified using the antibodies that were being tested.

4.3.1.1.2. Initial Monoclonal SLPI Primary Antibodies: No SLPI Detection in Saliva

Two different primary SLPI antibodies were initially run with the saliva samples. Both were monoclonal mouse IgG1, anti-human, antibodies (clone 31 from Abcam, UK and clone 20409 from R&D systems, UK). Although rSLPI was consistently detected, SLPI was not detected in the saliva samples using either antibody. Only clone 20409 was able to detect SLPI in the concentrated saliva cell pellets.

4.3.1.2. SLPI Primary Polyclonal Antibody: Successful Detection of SLPI in Various Oral Specimens

There was success with using a SLPI primary goat polyclonal antibody (0.5 mg/ml stock concentration from R&D System, UK) which was run with the donkey, anti-goat secondary antibody (R&D System) listed above in **Table 4.3.1.1.1.1**. SLPI was detected in several oral specimens using this antibody combination on the WB membrane seen in **Fig 4.3.1.2.1**.

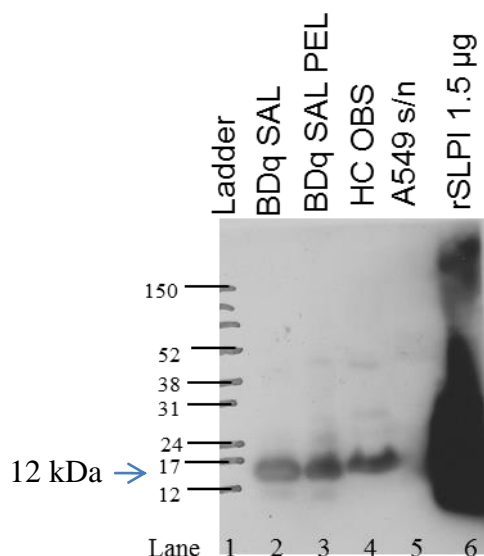


Figure 4.3.1.2.1. Detecting SLPI in Different Oral Specimens with the Polyclonal SLPI Primary Antibody (R&D Systems). Lane 1: Full Range Rainbow Ladder (GE Healthcare, UK). Lane 2: BD quiet (q, no oral ulcer) saliva, 14 µg total protein. Lane 3: BDq saliva cell pellet (SAL PEL) resuspended in 0.5 ml PBS, 9 µg added to the well. Lane 4: Health control (HC) oral buccal swab (OBS) total protein extract, 33 µg. Lane 5: SLPI-generating lung epithelial cell line (A549) supernatant (s/n). Lane 6: Recombinant (r)SLPI, 1.5 µg (proved to be too concentrated). The membrane was incubated at 1:500 dilution of polyclonal primary SLPI antibody (R&D Systems) and 1:3000 of the secondary donkey, anti-goat (R&D Systems). The band believed to be the SLPI protein was detected just above the expected 12 kDa.

The SLPI polyclonal antibody was specific and showed one clear SLPI band evident in the saliva pellet, saliva, and oral buccal swab samples. There was an issue with the rSLPI positive control which was extremely sensitive to the new antibody and components used for the ECL development and will therefore needed to be retested at a lower concentration. Unfortunately, it was also difficult to assess whether the A549 culture supernatant revealed a SLPI protein band as it was completely overshadowed by the concentrated rSLPI in the adjacent lane. However, blocking the PVDF membrane

overnight in 5% milk at 4°C improved detection of the SLPI protein in the patient samples as there was less background.

4.3.1.3. The Detection of SLPI in Saliva, Saliva Cell Pellets, and Oral Buccal Swabs from HC and BD Patients with and without Oral Ulcers

A full array of oral specimens which included saliva cell pellet, saliva, and oral buccal swabs from BDa, BDq, and HC subjects were run on the same WB. SLPI was detected just above the expected 12 kDa band (nearly at the 17 kDa marker) in all the oral samples (**Fig 4.3.1.3.1**). The rSLPI positive control in Lane 12a had DTT added to it (the main component in 10X NuPage reducing agent) which was not needed causing it to smear while running on the gel. This was rectified in future WB's (**Fig 4.3.1.3.1**. Lane 12b).

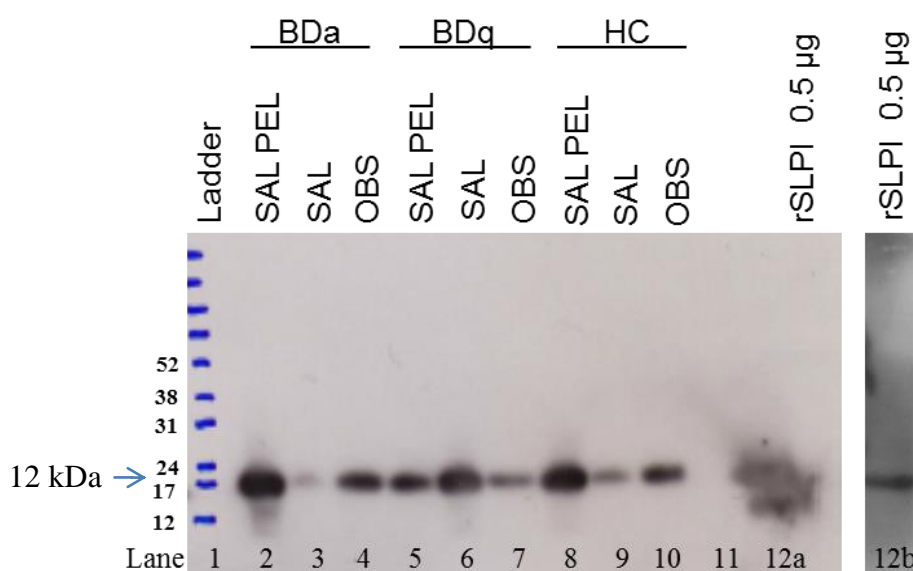


Figure 4.3.1.3.1. SLPI Detected in a Range of Oral Specimens from Three Patient Groups. Lane 1: Full Range Rainbow Ladder (GE Healthcare, UK). Lane 2,3,4: Resuspended saliva cell pellet (SAL PEL), saliva (SAL), and oral buccal swabs (OBS) from one BD active patient (a, having an oral ulcer). Lane 5,6,7: Resuspended saliva cell pellet, saliva, and oral buccal swabs from one BD quiet patient (q, no oral ulcer). Lane 8,9,10: Resuspended saliva cell pellet, saliva, and oral buccal swabs from one HC. Lane 11: Empty. Lane 12a: rSLPI, 0.5 µg with dithiothreitol (DTT) reducing agent. Lane 12b: rSLPI, 0.5 µg without DTT. Total protein was quantified for each saliva and OBS sample and 10 µg added to the well. Saliva cell pellets were not quantified prior to resuspension in 0.5 ml PBS, so a set volume of 8 µl was added to each designated well. The membrane was blocked overnight with 5% milk in PBS-0.05% Tween at 4°C after which it was incubated with 1:500 dilution of primary polyclonal SLPI antibody or 1 hour at RT followed by 1:3000 of the secondary donkey, anti-goat for 1 hour at RT. SLPI was detected just above the expected 12 kDa band (nearly at the 17 kDa marker) in all the oral samples.

4.3.1.4. Detection of NE in Oral Specimens

4.3.1.4.1. Detection of NE Using HRP-Labelled Primary Antibody

The next protein that needed to be detected in oral specimens was NE. A mouse IgG1 primary antibody was employed for this use on western blots, however the secondary antibody to couple with the primary was encountering the same non-specific binding issue as seen before. In order to circumvent the use of secondary antibodies because of the non-specific binding problems in saliva samples, purified NE monoclonal primary antibody was directly conjugated to a HRP label using Lightning-Link® (Innova Biosciences, UK).

Target	Antibody Details	Supplier/ Manufacturer	Dilution Used	Specific Binding?	Successful Detection of Target?
NE	Monoclonal primary Ab Mouse IgG1 Clone 265-3K1 Protein G purified Neat Concentration: 0.1 mg/ml	HyCult Biotech Cat # HM2174	1:200 and 1:1000	Yes	Yes, with additional bands (Fig 4.3.1.4.1.1)

Table 4.3.1.4.1.1. HRP Conjugated NE Primary Antibody. Details of the monoclonal NE primary antibody used for western blot detection of NE in oral specimens. The HRP (horseradish peroxidase) was chemically conjugated to the purified antibody eliminating the necessity of a secondary antibody. The HRP label allowed the target to be directly detected with electrochemical luminescence (ECL). Catalogue number (Cat #). IgG1, Immunoglobulin G1.

The NE-HRP antibody was successfully able to detect NE at the expected protein size 29.5 kDa in all saliva samples from BDa, BDq, and HC subjects (**Figure 4.3.1.4.1.1, A, B**). The protein bands beside the 31 kDa ladder marker on both **Fig 4.3.1.4.1.1** membranes **A** and **B** indicate the expected NE protein size which was also in line with the upper band of the positive control. Just as in previous SLPI western blots, the NE purified positive control was negatively affected with heat and the NuPage DTT reducing agent (Life Technologies, UK) as seen in **Fig 4.3.1.4.1.1, A, Lane 5** and so was not subjected to these treatments in subsequent experiments before loading onto the gels (**Fig 4.3.1.4.1.1, B, Lane 5**). Despite this precaution, the NE positive control still showed a lower band

around 12 kDa. After a consultation with the supplier, it was concluded that the NE protein purified from human peripheral blood neutrophils had some naturally-occurring degradation. The saliva samples from all the subjects also showed this degradation product around the same size. This was especially apparent in saliva from BDa patient in **Fig 4.3.1.4.1.1, A, Lane 2**. The protein isolated from the blood and oral buccal swab samples in **Fig 4.3.1.4.1.1, B** showed the same size 29.5 kDa NE band as the saliva but seemed to be separated into two thinner bands in close proximity to each other. This effect was not seen in any saliva samples.

In addition to this lower band, all saliva samples also showed a thick larger protein band between 52 and 76 kDa. This band was seen again in the whole blood protein (**Fig 4.3.1.4.1.1, B, Lane 2**) and the ulcerated oral buccal swab sample (**Fig 4.3.1.4.1.1, B, Lane 4**) but with less intensity. The saliva samples in **Fig 4.3.1.4.1.1, A** also had another band above 76 kDa. It is suspected that these bands indicated an unknown protein complex with NE or a contaminant with a similar NE epitope. The whole blood and oral buccal swab protein samples were isolated using Qiagen's DNeasy column kit (Qiagen, UK) followed by an acetone protein precipitation. Therefore, any complexes or contaminants could have been reduced in the blood and buccal samples. This could explain the lack of small degradation products in these processed samples as well. Meanwhile, the saliva samples did not undergo any protein isolation procedures before being loading onto the western blot gels and could have various NE complexes.

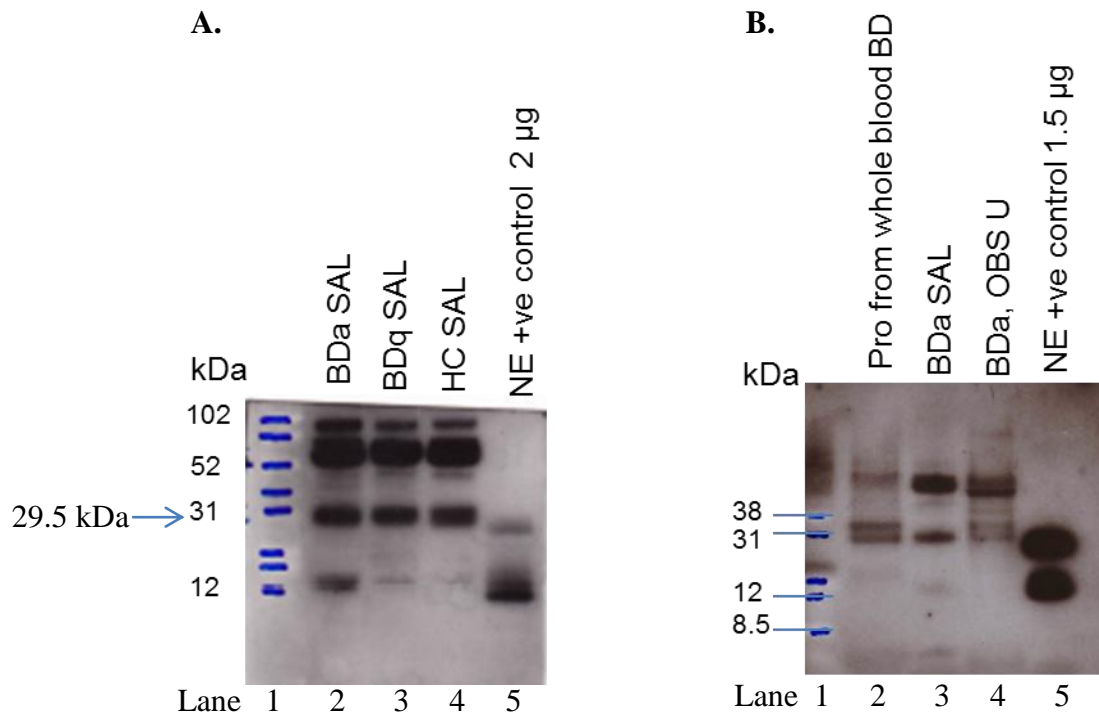


Figure 4.3.1.4.1.1. A and B. NE Detected in Saliva, Blood, and Oral Buccal Swab Samples. **A:** Saliva samples' total protein was quantified and 10 µg added to each well. Membrane was blocked with 5% milk overnight at 4°C before adding the 1:200 HRP-NE conjugated monoclonal primary antibody for 1 hour incubation at RT. Lane 1: Full Range Rainbow Ladder (GE Healthcare). Lane 2: BDactive (a, ulcer) saliva. Lane 3: BDquiet (q, no ulcer) saliva. Lane 4: HC saliva. Lane 5: NE positive (+ve) control, 2 µg, with dithiothreitol (DTT) reducing agent. **B:** Membrane was blocked with 5% goat serum overnight before adding 1:500 NE-HRP primary antibody for 1 hour incubation at RT. Lane 1: Low Range Rainbow Ladder. Lane 2: 5 µg of isolated protein from whole blood from BDq (no ulcer, but had active folliculitis and joint pain). Lane 3: BDa saliva, 10 µg. Lane 4: BDa oral buccal swab (OBS) taken directly from an ulcer (U), 10 µg. Lane 5: NE +ve control, 1.5 µg, no reducing agent (DTT) or heat denaturing.

4.3.1.5. Detection of α 1AT in Saliva

Saliva was also tested for the levels of α 1AT in BD and RAS patients in comparison to HC. Previous literature indicated that α 1AT in saliva from periodontitis patients could exceed that of NE (Pederson et al., 1995), so the saliva from our patients was diluted 1:200 and 1:600 before being run on the western blot. The expected 52 kDa α 1AT protein band was successfully detected in BDa, BDq, and HC saliva and was the same size as the positive control. The amount of α 1AT in BDa saliva was still quite high causing dark streaks on the WB (**Fig 4.3.1.5.1, Lane 2, 3**), but was reduced in BDq (**Lane 4, 5**) and was not an issue in HC saliva (**Lane 6, 7**).

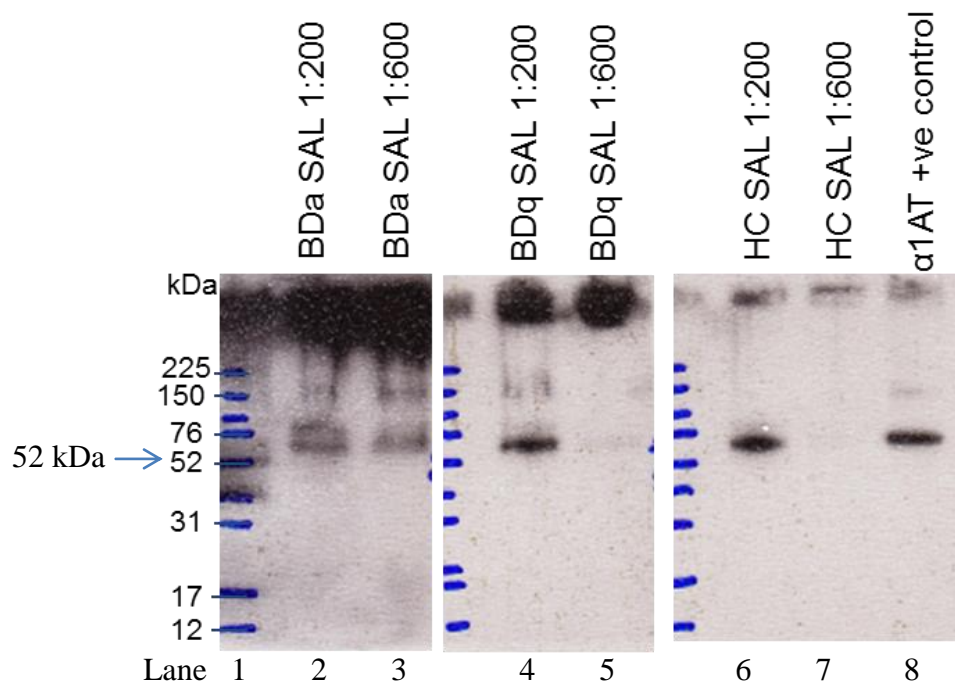


Figure 4.3.1.5.1. α 1AT Detected in Saliva Samples. Saliva samples' total protein was quantified and 0.03 μ g added to each well. Membrane was blocked with 5% milk overnight at 4°C before adding 1:1000 rabbit, anti-human α 1AT polyclonal primary antibody for 1 hour incubation at RT followed by 1:1500 goat, anti-rabbit HRP secondary antibody. Lane 1: Full Range Rainbow Ladder. Lane 2: BDactive (a, ulcer) saliva (SAL) diluted 1:200. Lane 3: Same BDa saliva diluted 1:600. Lane 4: BDquiet (q, no ulcer) saliva diluted 1:200. Lane 5: Same BDq saliva diluted 1:600. Lane 6: HC diluted 1:200. Lane 7: Same HC diluted 1:600. Lane 8: Isolated total protein from lysed A549 lung epithelial cell line used as α 1AT positive (+ve) control, 0.15 μ g.

4.3.2. Measurement of Salivary Levels of NE, SLPI, and Alpha-1 Antitrypsin using ELISA

In order to determine quantitatively if there was an excess of NE present in BD and RAS patients' saliva during quiet, non-ulcerative episodes, ELISAs were used to quantify the protein. Importantly, saliva NE was also tested during an active oral ulcer to see if BD and RAS patients would have similar levels due to their innate neutrophil response to a wound. These values were compared to HCs. SLPI and α 1AT were also measured in all the same patient groups using ELISA to determine if there was a direct quantitative relationship between NE and its inhibitors in saliva. The number of saliva samples tested in each patient group is summarised in **Table 4.3.2.1**.

Patient Group	NE n	SLPI n	α 1-AT n
BDa	49	52	32
BDq	59	56	29
HC	54	49	19
RASq	10	11	5
RASa	12	15	4

Table 4.3.2.1. Patient Saliva Sample Number for Each ELISA. The number of patient saliva samples (n) that were measured by ELISA for each target: NE, SLPI, and α 1AT.

4.3.2.1. The Levels of NE, SLPI, and α 1AT in Saliva from HC, BD and RAS Patients

Statistical analysis showed that BDa saliva (1794 ng/ml, group median value) had significantly higher levels of NE than HC (998.9 ng/ml, $p < 0.01$) and BDq (1111.7 ng/ml, $p < 0.05$) (**Fig 4.3.2.1.1**). There was also a 2-tailed significant difference, $p < 0.05$, between the BDa patient group and RASq (1043.4 ng/ml). RASa saliva (3080.7 ng/ml) had the highest NE levels which was particularly evident when compared to HC ($p \leq 0.001$), RASq ($p < 0.01$), and BDq ($p < 0.01$). HC showed the lowest levels of NE.

The median NE level measured in the BDq patient group was higher than HC and RASq, but this was not statistically significant. However, when considering the protein quantification range, BDq saliva had a much larger range and higher maximum value than either HC or RASq. The minimum NE measurement in BDq saliva was 132.7 ng/ml while the maximum was 10,124.1 ng/ml. Meanwhile, HC NE levels ranged from 196 - 5,178 and RASq from 287.3 – 2,673.4 ng/ml. **Table 4.3.2.3** outlines these minimum and maximum values as well as the 25th and 75th percentile range.

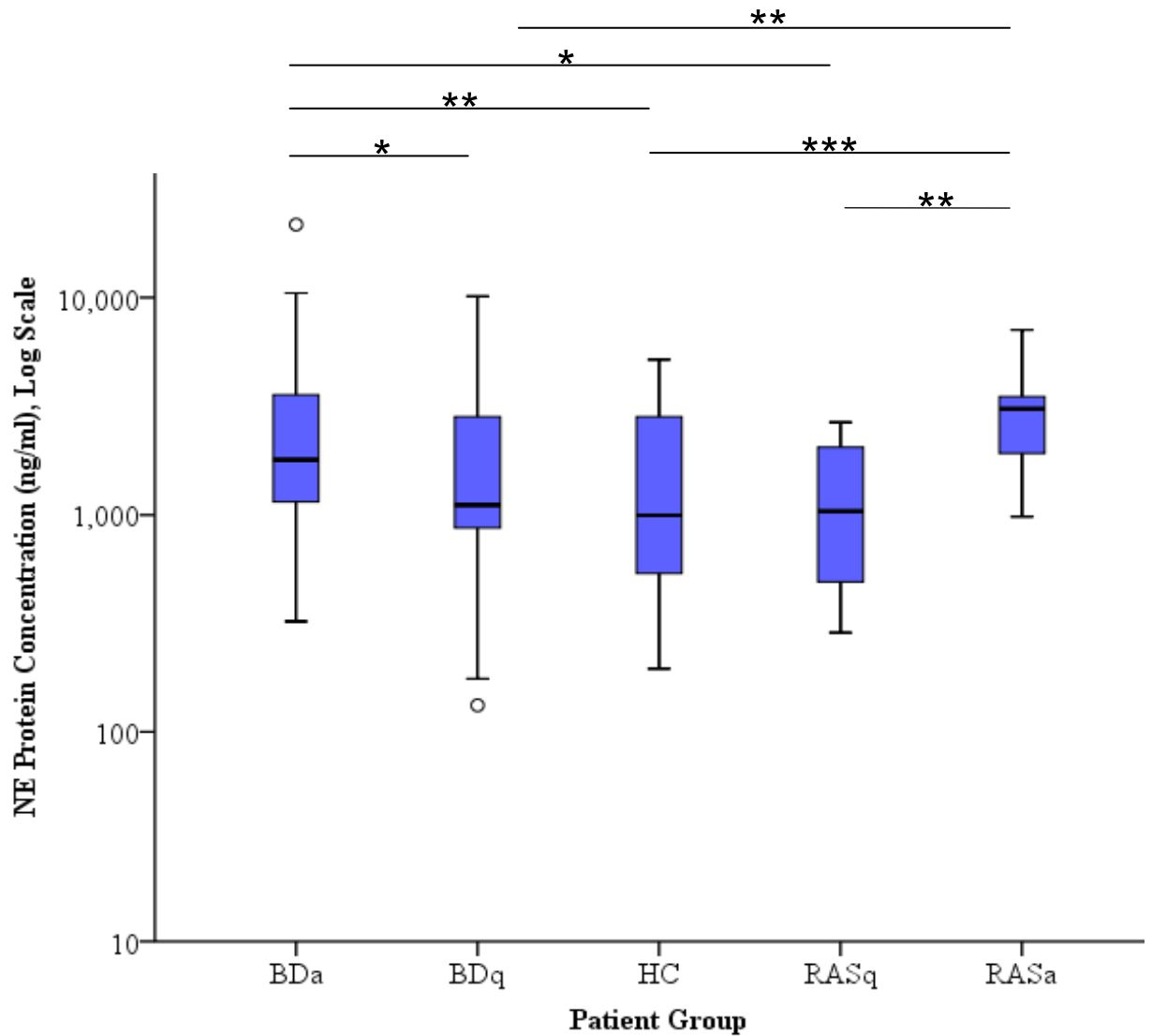


Figure 4.3.2.1.1. Salivary NE levels amongst different patient groups. The black centre bar in each box of the plot represents the median NE protein level measured for each group. The box itself represents 50% of the values in the group and the extended bars indicate the extent of maximum and minimum values therefore encompassing the range. **o** indicates outliers. Data is plotted on a logarithmic scale. **HC** (healthy control), **RAS** (recurrent aphthous stomatitis), **BD** (Behçet's disease). **q**, indicates mouth quiet/no ulcers. **a**, refers specifically to active mouth ulcer(s) regardless of other systemic symptoms. The non-parametric Mann-Whitney U test was used to assess significant difference between two groups. Significance based on 2-tailed, 95% CI. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

SLPI levels were found to be the highest in RASq (Figure 4.3.2.1.2). When comparing group medians, the level of SLPI was significantly higher in RASq (860.1 ng/ml) than BDq (198.7 ng/ml) and BDa (320.6 ng/ml) patient groups, $p < 0.01$. The 487.3 ng/ml of SLPI in HC saliva was also significantly higher than BDq, $p < 0.05$. BDq had the lowest levels amongst all the other groups.

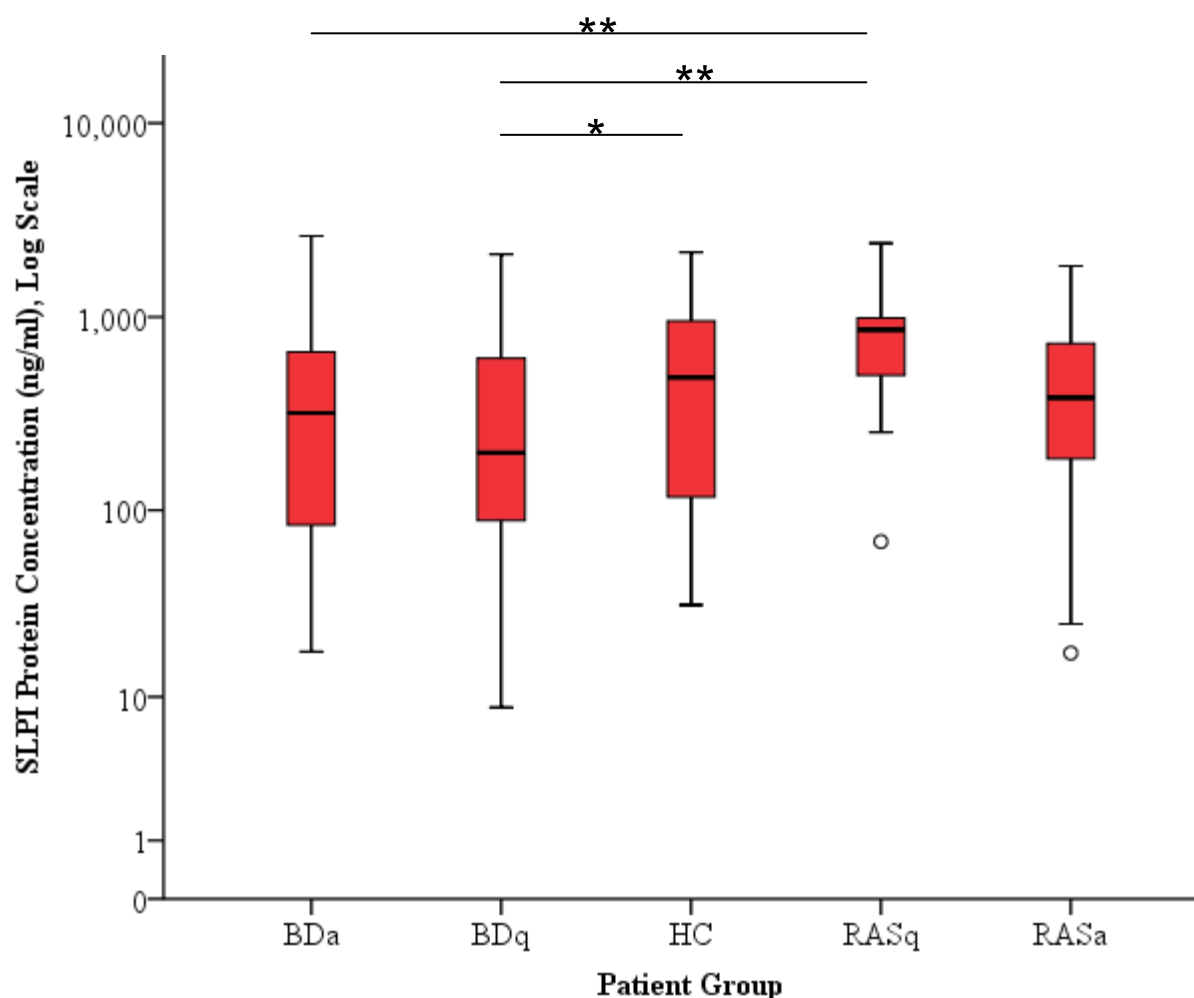


Figure 4.3.2.1.2. Salivary SLPI levels amongst different patient groups. The black centre bar in each box of the plot represents the median SLPI protein level measured for each group. The box itself represents 50% of the values in the group and the extended bars indicate the extent of maximum and minimum values therefore encompassing the range. **o** indicates outliers. Data is plotted on a logarithmic scale. **HC** (healthy control), **RAS** (recurrent aphthous stomatitis), **BD** (Behçet's disease). **q**, indicates mouth quiet/no ulcers. **a**, refers specifically to active mouth ulcer(s) regardless of other systemic symptoms. The non-parametric Mann-Whitney U test was used to assess significant difference between two groups. Significance based on 2-tailed, 95% CI. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Since NE is also regulated by α 1-AT, the levels α 1-AT inhibitor was also measured (**Figure 4.3.2.1.3**). Salivary levels of α 1-AT were significantly higher in BDa (14,326.1 ng/ml) than HC (4995 ng/ml) ($p < 0.001$) while BDq (8395.3 ng/ml) was significantly higher than HC ($p < 0.05$) as well. Most of NE, SLPI and α 1-AT measurements were carried out on the matching samples taken from each patient. An inverse relationship between SLPI and NE was observed when comparing the concentration of the two targets between patients groups (**Figure 4.3.2.1.4**). Analysis showed that when the concentration of SLPI in saliva was low, the concentration of NE was high and vice versa. For instance, BDa had a high NE concentration of 1794 ng/ml while SLPI resulted in a low median value of 320.6 ng/ml. This suggested that there is either a depletion of SLPI in saliva when NE is increased or that SLPI is binding to NE or another substrate thereby decreasing its epitope availability for antibody detection. This did not seem to be an issue with the ELISA measurement of NE and α 1-AT as these proteins showed high quantitative levels and had a direct relationship with each other (**Figure 4.3.2.1.4**).

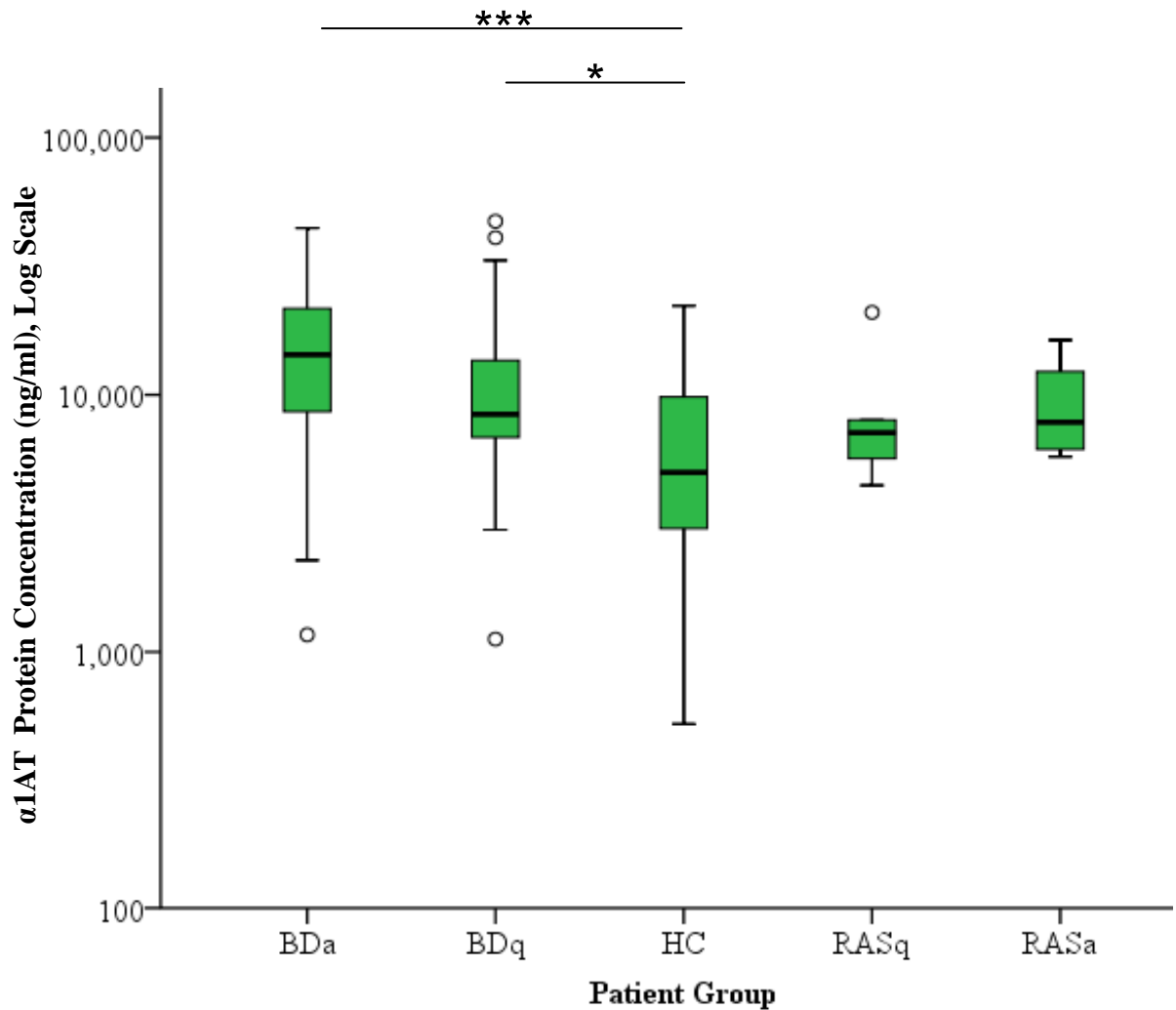


Figure 4.3.2.1.3. Salivary α1AT levels amongst different patient groups. The black centre bar in each box of the plot represents the median α1AT protein level measured for each group. The box itself represents 50% of the values in the group and the extended bars indicate the extent of maximum and minimum values therefore encompassing the range. **o** indicates outliers. Data is plotted on a logarithmic scale. **HC** (healthy control), **RAS** (recurrent aphthous stomatitis), **BD** (Behçet's disease). **q**, indicates mouth quiet/no ulcers. **a**, refers specifically to active mouth ulcer(s) regardless of other systemic symptoms. The non-parametric Mann-Whitney U test was used to assess significant difference between 2 groups. Significance based on 2-tailed, 95% CI. * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$.

It was also noteworthy that the concentration of the NE inhibitor α1-AT in the saliva far exceeded that of NE and SLPI which is demonstrated in **Fig. 4.3.2.1.4**. The medians for each patient group are compared in **Table 4.3.2.2**.

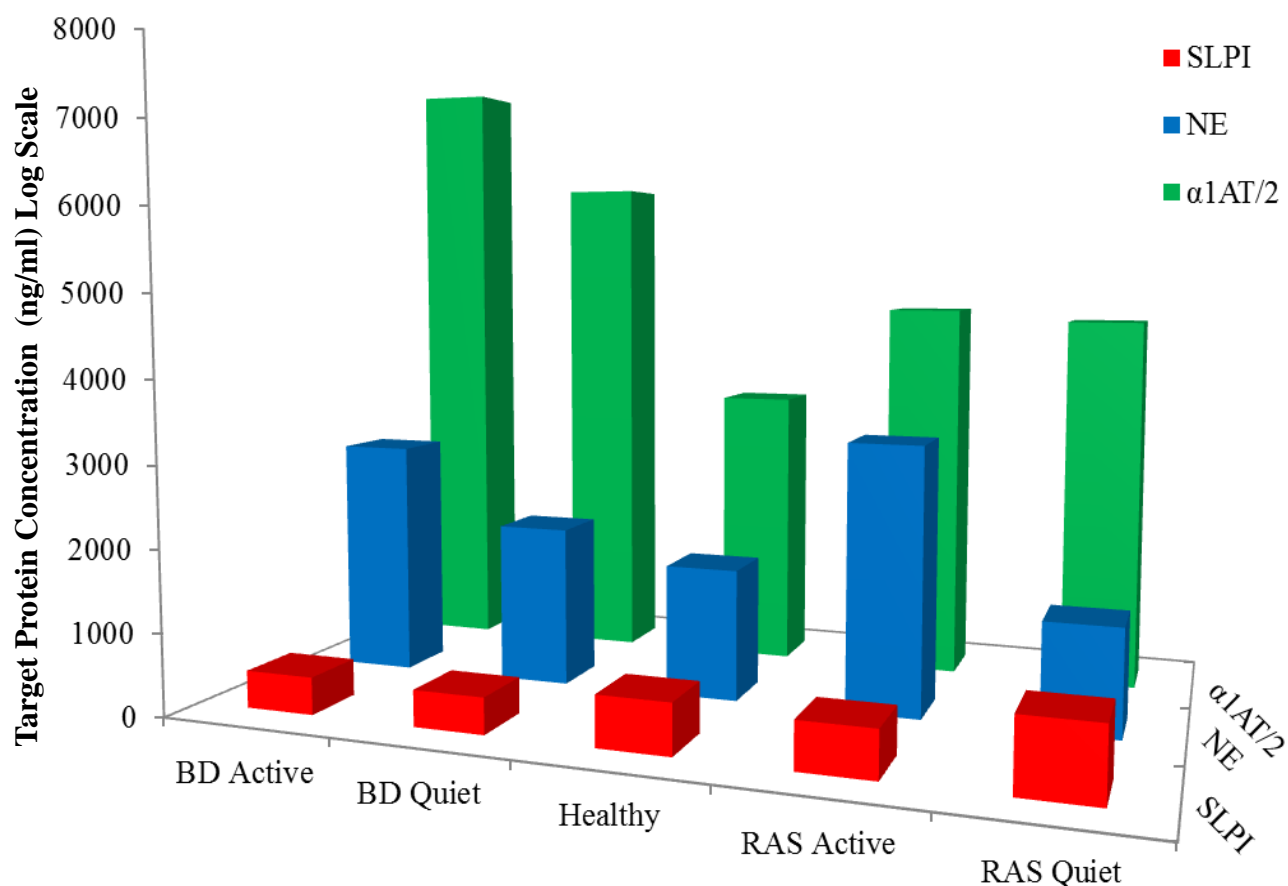


Figure 4.3.2.1.4. Comparison of SLPI, NE and Alpha1-Antitrypsin. An alternative depiction of Figures 4.3.2.1.1, 4.3.2.1.2, and 4.3.2.1.3 showing the differences in concentrations between NE and its inhibitors in saliva. Note that α 1AT is graphed at half of its concentration measured by ELISA.

Protein Target	BD Active (ng/ml)	BD Quiet (ng/ml)	Healthy (ng/ml)	RAS Active (ng/ml)	RAS Quiet (ng/ml)
SLPI	320.59	198.72	487.28	383.01	860.09
NE	1793.95	1111.71	998.87	3080.73	1043.35
α 1AT	14326.07	8395.34	4994.96	7934.22	7118.54

Table 4.3.2.2. Patient Groups Median Concentration Values of NE, SLPI, and α 1AT Salivary Protein Targets Measured by ELISA. These group median values are demonstrated in Figures 4.3.2.1.1, 4.3.2.1.2, and 4.3.2.1.3).

Protein Target and Range Details	Patient Group and Mouth Activity				
	BD Active (ng/ml)	BD Quiet (ng/ml)	Healthy (ng/ml)	RAS Active (ng/ml)	RAS Quiet (ng/ml)
NE 25 th percentile	1148.92	872.39	544.48	2194.29	566.47
NE 75 th percentile	3573.57	2832.66	2800.84	3461.04	1975.79
NE Min Value	323.59	132.77	195.98	983.64	287.32
NE Max Value	21623.60	10124.09	5178.03	7106.02	2673.44
SLPI 25 th percentile	83.98	92.04	117.27	185.90	512.35
SLPI 75 th percentile	650.32	608.50	954.42	732.15	992.08
SLPI Min Value	17.79	8.70	31.81	17.52	68.52
SLPI Max Value	2616.33	2107.81	2159.66	1831.42	2404.99
α 1AT 25 th percentile	8609.95	6823.78	3017.83	6365.33	5656.08
α 1AT 75 th percentile	21286.10	13618.59	9810.08	11055.11	14890.83
α 1AT Min Value	1165.46	1121.41	524.77	5739.36	4448.71
α 1AT Max Value	44454.82	47313.33	22174.37	16337.12	20931.38

Table 4.3.2.3. Value Range of the NE, SLPI, and α 1AT Salivary Protein Targets Measured by ELISA. The 25th and 75th interquartile values as well as the minimum (min) and maximum (max) protein concentration values in ng/ml units are listed above. These values are represented in the box plots in Figures 4.3.2.1.1, 4.3.2.1.2, and 4.3.2.1.3.

In order to confirm the very high α 1AT levels found in saliva, an additional α 1AT ELISA was conducted on patient serum which is the main constituent for delivery of α 1AT to the tissues. These samples were diluted in PBS at 1:100, 1:10,000, and 1:100,000 and measured. The 1:100,000 serum dilution generated the best assessment of α 1AT content (see **Table 4.3.2.3.4**). This range was compared to those in published literature (i.e. for the HC group since BD and RAS patients have not previously had this inhibitor measured in their saliva). The range of α 1AT was confirmed to be within an accurate range. Therefore, this provided additional confirmation that the high α 1AT values measured in the saliva samples using ELISA were accurate.

Patient Group and Oral Ulcer Activity	n	Group Median ng/ml	Range (Min and Max) (ng/ml)	
HC	5	1.94×10^6	1.63×10^6	2.48×10^6
BDq (collective)	5	1.98×10^6	1.77×10^6	2.46×10^6
BDq, no symptoms	2	1.87×10^6	1.77×10^6	1.98×10^6
BDq, other symptoms	3	2.09×10^6	1.79×10^6	2.46×10^6
BDa	4	2.0×10^6	6.9×10^5	2.92×10^6
RASa	1	n/a	n/a	n/a
RASq	2	2.32×10^6	2.03×10^6	2.61×10^6

Table 4.3.2.3.4. α 1AT Levels in Serum. Serum samples were measured using the same α 1AT ELISA as saliva samples for comparison. **n**, number of subjects' serum tested. **Min**, minimum values. **Max**, maximum values.

4.3.2.2. NE, SLPI, and α 1AT Saliva Concentrations From BD Patients Taking Colchicine

Colchicine is one of the recommended medications for oral ulcers in BD (Sander and Randle, 1986). It works by reducing the migration of neutrophils (Roubille et al., 2013). Therefore, NE and its inhibitors' α 1AT and SLPI concentrations were assessed in saliva from BD patients taking colchicine and whether they had oral ulcers.

There were significant differences when comparing the group median concentrations in NE. BDq patients taking colchicine still had significantly high NE in their saliva compared to BDq who were not on the medication ($p=0.036$) (**Fig 4.3.2.2.1**). This suggests that colchicine is not reducing the recruitment of oral neutrophils and therefore the presence of NE in the oral cavity during quiescent ulceration. The BDq group not taking colchicine had significantly lower NE than both BDa on colchicine ($p=0.009$) or not on colchicine ($p=0.028$). Taking colchicine during an active ulcer was able to reduce the concentration of salivary NE but not significantly.

Although none of the α 1AT and SLPI level comparisons were significant amongst BD patients on colchicine, there were notable trends. BDa patients on colchicine showed increased α 1AT suggesting that colchicine may help increase the quantity of α 1AT in saliva for protection against NE. Meanwhile, BDa patients not taking colchicine had a rise in SLPI concentration. This is surprising since the usual trend amongst our patient population was to see a decrease of SLPI during active oral ulceration.

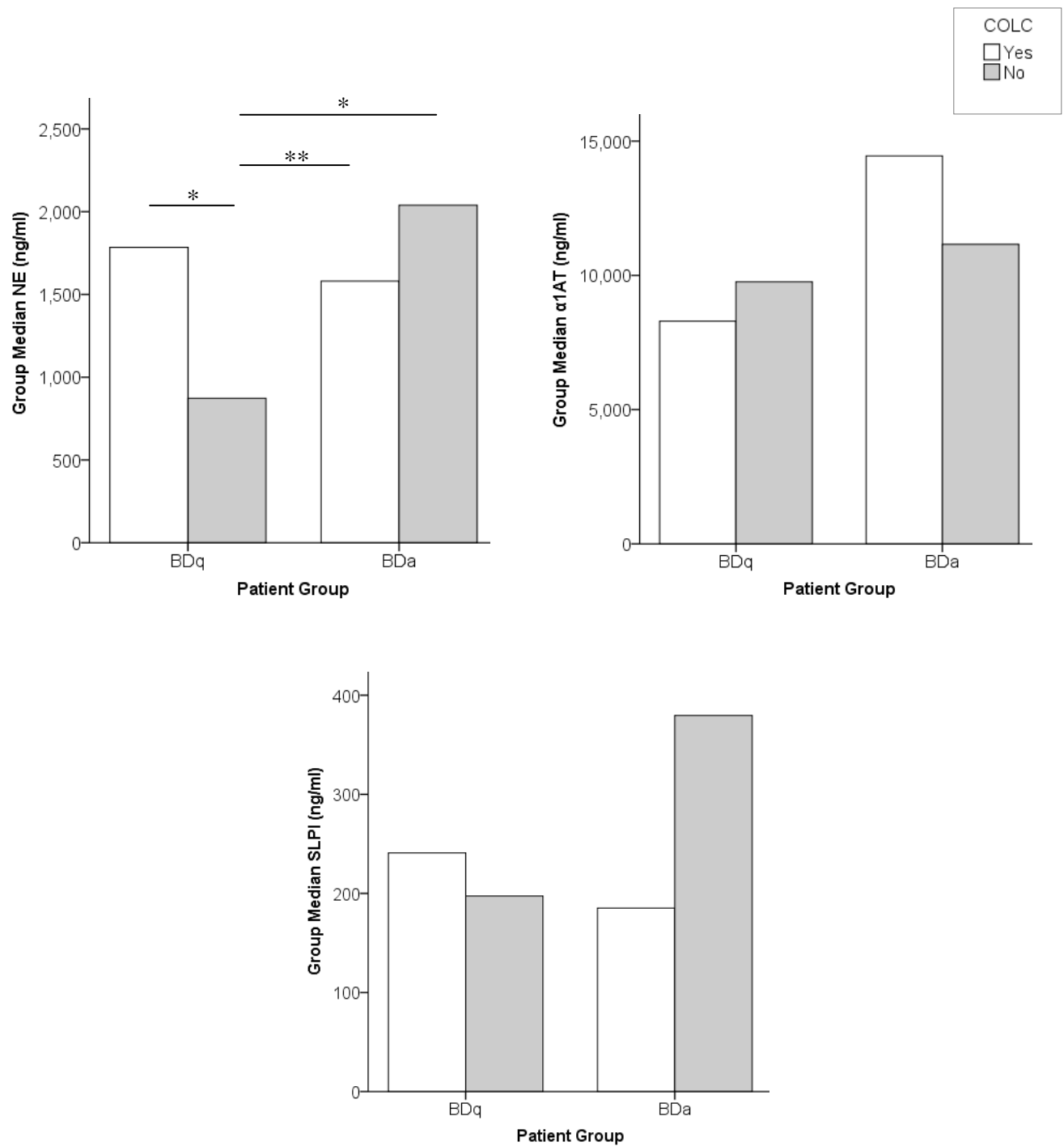


Fig 4.3.2.2.1. Comparing Saliva NE, α 1AT, and SLPI Concentrations from BD Patients Taking Colchicine. The bars graphs show the group median NE, α 1AT, and SLPI saliva concentrations from BD Behçet's Disease (BD) quiet (q, no oral ulcers) and BD active (a, oral ulcer) patients. *p<0.05. **p<0.01.

4.3.3. NE and SLPI in Western Blots: Comparing ELISA Measurements

To investigate the indirect relationship seen in NE and SLPI protein levels measured by ELISA, some of the same saliva samples were run on western blots. This was to see if the quantitative ELISA measurements would coincide with the protein band intensity of NE and SLPI. **Fig. 4.3.3.1, A and B** confirmed that the intensity of SLPI and NE protein bands detected on the western blots corresponded to the ELISA levels. One SLPI band is evident in the SLPI membrane (**Fig. 4.3.3.1, A**). However, the majority of saliva samples on the NE western blot (**Fig. 4.3.3.1, B**) again revealed an additional upper (52 – 76 kDa) and lower band (just above 12 kDa) accompanying the expected 29.5 kDa NE.

Considering that the primary SLPI antibody used in the western blot and the ELISA antibodies are from the same company (R&D Systems, UK), it is possible that they are only binding to un-bound or “free” SLPI. This would coincide with the single protein band at 12 kDa on the western blot. Meanwhile, the NE antibody could be detecting all bound, un-bound and fragmented NE explaining the multiple protein bands.

Human oral keratinocyte protein (purified from an *ex vivo* culture) was included as a negative control and was not expected to show any NE band at 29.5 kDa which indeed was absent. However, an upper band just above 52 kDa did appear at the same size as the large, unknown protein seen in the saliva samples strongly suggesting that this upper band could be a protein contaminant from the foetal calf serum used in the culture media which would contain NE, SLPI, or α 1AT.

SEE FIGURE 4.3.3.1.

Figure 4.3.3.1. SLPI and NE in Saliva. The total protein was quantified and 10 µg loaded per well. **A.** Polyclonal SLPI antibody was diluted 1:500 and incubated for 1 hr at RT. After washing, a donkey, anti-goat secondary antibody was diluted to 1:3000 and incubated with the membrane for 1 hr at RT. Lane 1: Rainbow Full Range Protein Marker (GE Healthcare). Lane 2: BDa (active ulcer) saliva. Lane 3: BDq (quiet, no ulcer) saliva. Lane 4: RASa saliva. Lane 5: RASq saliva. Lane 6: HC saliva. Lane 7 and 8: recombinant SLPI (rSLPI, 12-14 kDa). **B.** Monoclonal NE-HRP antibody was diluted 1:500 and incubated for 1 hr at RT. Lane 7 and 9: purified human NE, 29.5 kDa, positive control (NE+), no DTT added or heat treatment. Lane 8: Empty. Lane 10: Oral keratinocyte (Oker) isolated culture supernatant as a NE negative control.

4.3.4. Total Protease and Specific NE Enzyme Activity in Saliva

Serine proteases, like NE, have the ability to cleave protein substrates after small hydrophobic residues (such as valine) and are characterised by an active serine amino acid site (Heutinck et al., 2010). Two reaction systems were utilised to assess the activity of both endogenous NE and purified NE. The first employed the use of the fluorometric EnzChek[®] Elastase Assay kit (Molecular Probes) to prove not only that active NE was present in a patient saliva by its specific inhibition with *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (**Fig 4.3.4.2.1** to **Fig 4.3.4.2.2**), but also that total protease activity could be determined by the breakdown of fluorescent-conjugated bovine elastin. The second system tested the ability of purified NE (Abcam, UK) and endogenous, i.e. native, NE in saliva to degrade a colorimetric human elastin substrate, MeOSuc-Ala-Ala-Pro-Val-pNA (Sigma, UK), coupled with a potent specific NE inhibitor, GW311616A (Sigma, UK).

4.3.4.1. Proof of Concept: Both Recombinant SLPI and a Chloromethyl Ketone Specific Inhibitor Can Prevent Purified NE from Hydrolysing Bovine Elastin Substrate

Initially, a proof of concept experiment was conducted in order to show that purified NE could be inhibited by purified rSLPI. Enzyme and inhibitor were combined and tested at variable dilutions alongside a constant concentration of 25 µg/ml fluorescent-conjugated bovine elastin. The lower of the two rSLPI concentrations tested, 1.08 µM, was able to successfully inhibit the majority of 0.385 µM (0.045 U/rxn) of NE resulting in a NE:SLPI concentration ratio of about 1:3 (**Fig. 4.3.4.1.1**). The same concentration of 0.385 µM NE was completely inhibited by the NE specific inhibitor 75 µM of *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone in a control reaction. However, when the NE concentration was increased to 0.424 µM (0.05 U/rxn), 1.08 µM of SLPI was not sufficient to inhibit the NE activity. Double the concentration of rSLPI (2.14 µM) was able to provide some inhibition of a larger concentration of NE (0.848 µM (0.1 U/rxn)). The highest NE concentration, 0.848 µM, was tested with 1.08 µM of rSLPI resulting with the NE in the reaction immediately digested nearly 100% of the elastin available, producing the largest baseline fluorescent measurement (**Fig 4.3.4.1.1**). When using the porcine pancreatic elastase, both concentrations of rSLPI were not as effective in inhibiting the digestion. However, the higher concentration of rSLPI was able to inhibit the porcine elastase more efficiently. A positive control reaction was run at the same time

whereby porcine elastase (0.01 U/rxn) was 100% inhibited by the ketone inhibitor (75 μ M) throughout the 95 min, 30°C reaction.

The majority of the rSLPI inhibition occurred within the first 5 min of each reaction. This was demonstrated by either no change or a slight increase of fluorescence (**Fig 4.3.4.1.1**). Most of the elastin degradation by NE occurred between 5 and 45 min in all reactions since the largest fluorescence increase was seen during this period.

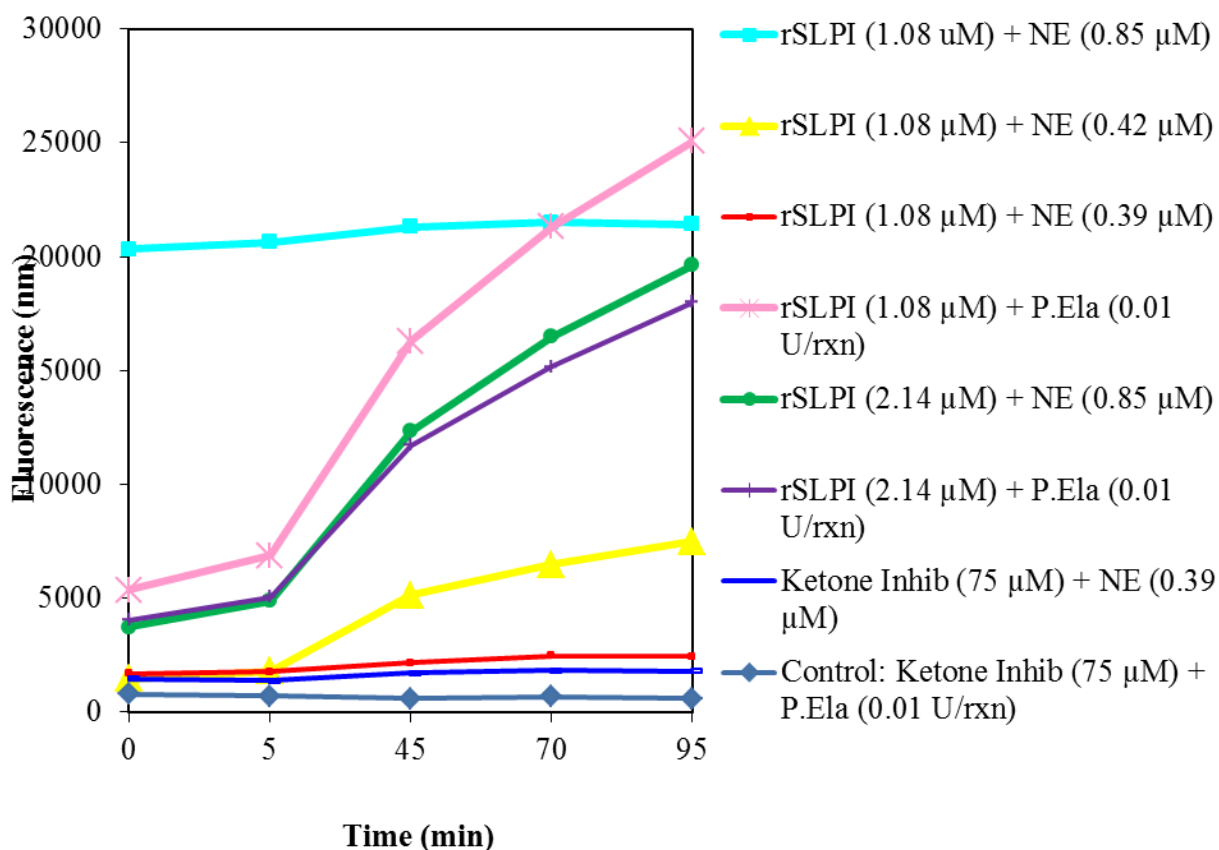


Fig. 4.3.4.1.1. The Inhibitory Activity of rSLPI on the Enzyme Activity of NE: Testing Purified Proteins. A validation experiment was completed to test different concentrations of recombinant SLPI protein (rSLPI) and its ability to inhibit both purified neutrophil elastase (NE) and porcine pancreatic elastase (P.Ela.) in the presence of 25 μg/ml fluorescent-conjugated bovine elastin. All proteins were commercially purchased. A combination of NE and *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (ketone inhib) was also used as a control for inhibiting NE activity. A negative control was also tested combining known inhibitory concentration of ketone inhibitor for P.Ela. A constant fluorescence (no change) indicates efficient inhibition. Increased fluorescence denotes that the reaction is not being inhibited effectively; however a small difference in change of fluorescence from the start of the assay to the end indicates some inhibition. Blank wells with only reaction buffer and fluorescence substrate were measured in order to deduct background fluorescence from the samples and controls. All reactions were tested in duplicate.

4.3.4.2. Total Protease Activity and Specific NE Activity in Saliva: Initial Tests

Both experiments in **Fig 4.3.4.2.1** and **Fig 4.3.4.2.2** tested the same BDa saliva sample. After 95 minutes, the difference between 25 μM and 150 μM of inhibitor in a 1:8 saliva dilution showed only a 46% decrease of NE activity (**Fig 4.3.4.2.1**). Larger saliva dilutions (up to 1:40) were tested with 25 μM of inhibitor (**Fig 4.3.4.2.2**). It was found that NE activity decreased by 89% by diluting the final saliva concentration from a 1:4 to a 1:40 dilution. But again, 100% inhibition was not achieved in the samples. The neat salivary NE content, 1347 ng/ml, had previously been measured by ELISA. At a 1:40 dilution, the NE level would be about 33.7 ng/ml (or 5.71 μM) in the reaction. In order to fully inhibit the NE, either the saliva will have to be diluted 11% more or the inhibitor concentration will have to be increased by 11%. As a negative control, saliva alone was tested for auto-fluorescence, but it was confirmed saliva did not emit any fluorescence (data not shown).

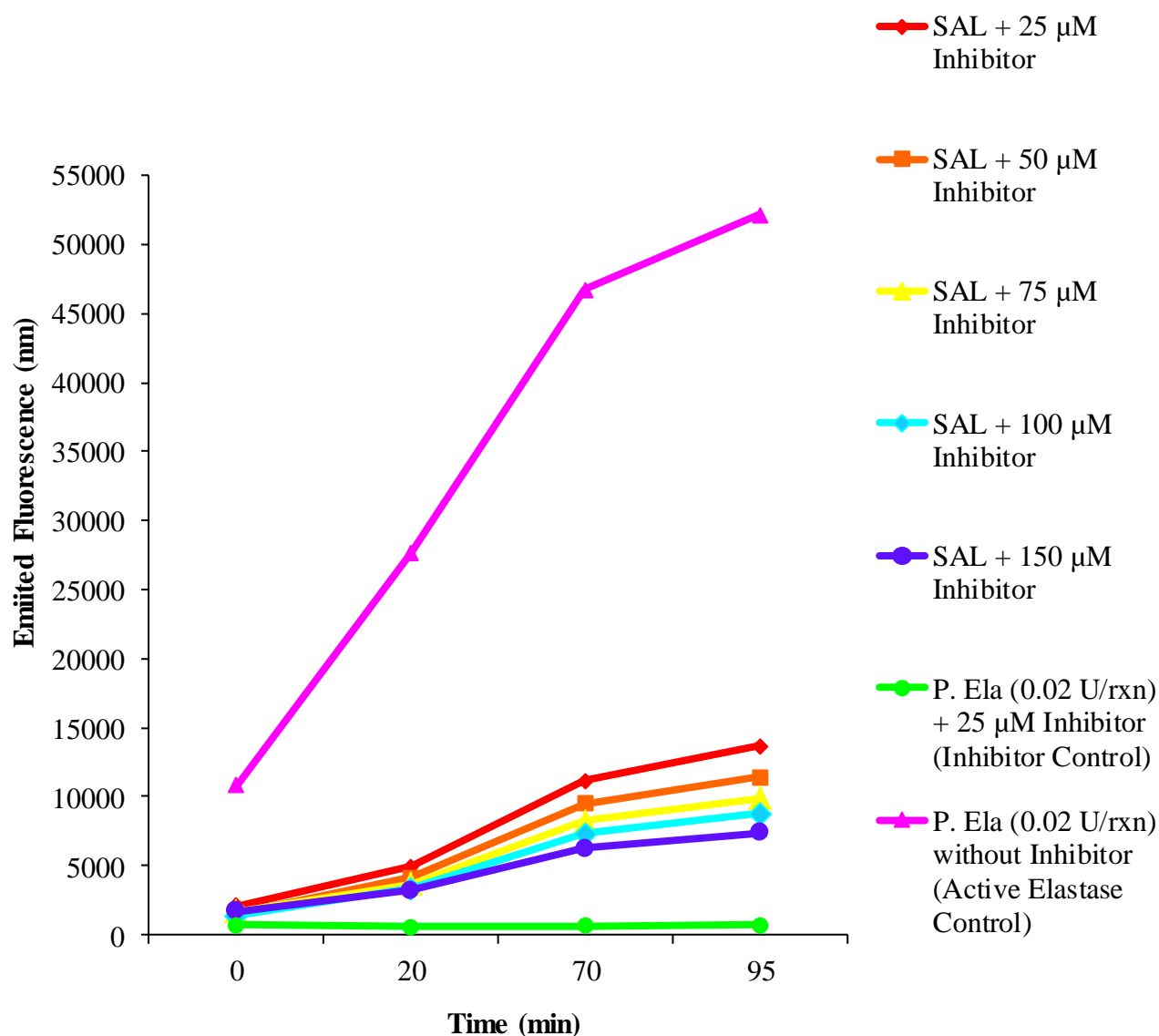


Figure 4.3.4.2.1. Specific Inhibition of Endogenous NE in BDa Saliva: Testing Different Concentrations of Inhibitor (a Range of 25-150 μ M) with a BDa Saliva Sample. The specific chloromethyl ketone inhibitor was added at various concentrations to a 1:8 saliva (SAL) dilution (this was the final dilution of saliva in the reaction). Porcine elastase (P.Ela., 0.02 units per reaction, U/rxn) with and without 25 μ M inhibitor were used as controls. The digestion of bovine elastin was monitored by the release of its conjugated fluorescence during a 95 minute incubation. A high inhibitor concentration (>150 μ M) was required to inhibit only part of the neutrophil elastase activity in a 1:8 diluted saliva sample.

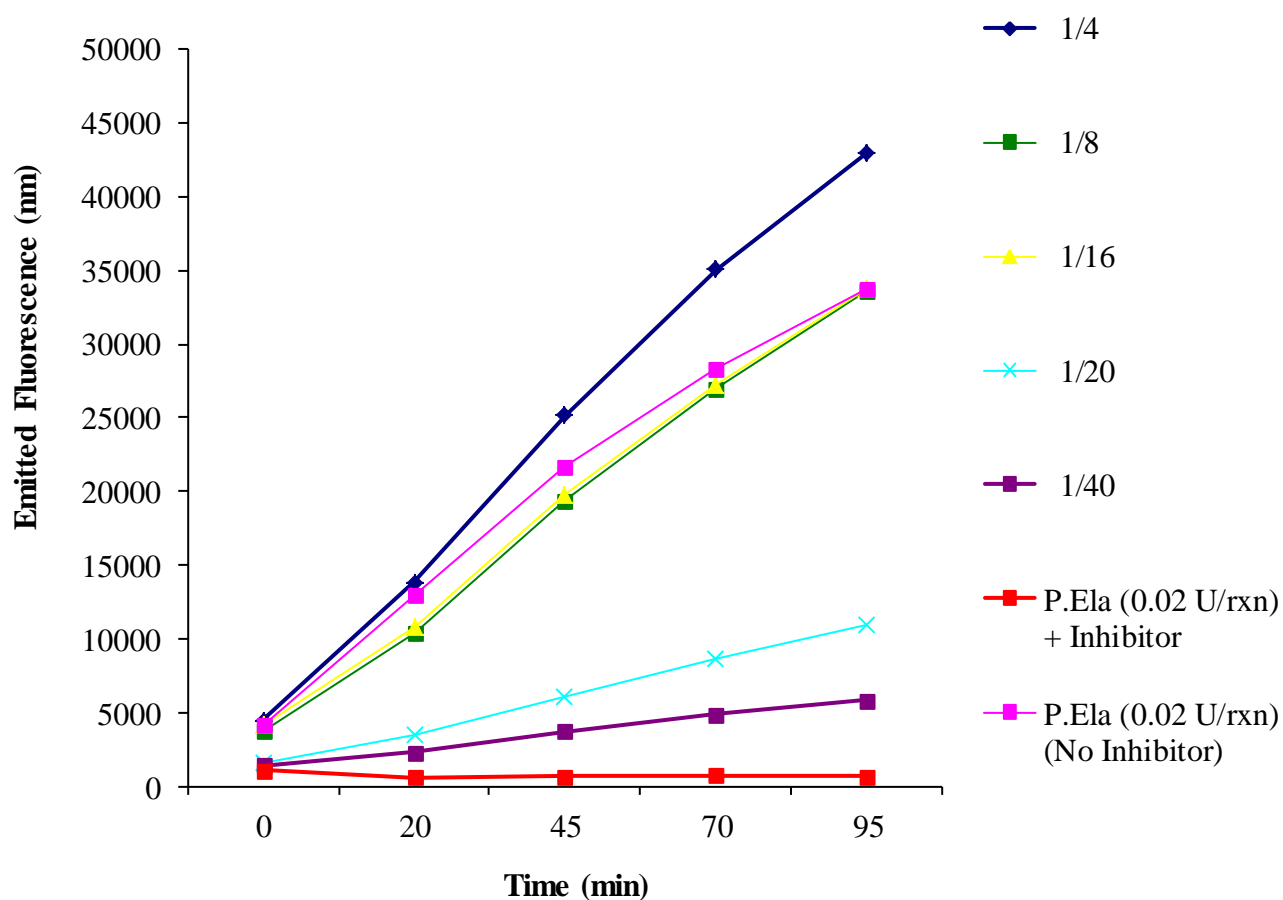


Figure 4.3.4.2.2. Specific Inhibition of Endogenous NE in BDa Saliva: Testing One Concentration of Inhibitor (25 μ M) with One BDa Saliva Sample at a Various Final Dilutions (Range 1:4 to 1:40). The specific chloromethyl ketone inhibitor was added at 25 μ M to the various saliva dilutions (in the final reaction). Porcine elastase (P.Ela., 0.02 units per reaction, U/rxn) with and without 25 μ M inhibitor were used as controls. The digestion of bovine elastin was monitored by the release of its conjugated fluorescence during a 95 minute incubation.

4.3.4.3. Total Protease Activity in Saliva: Comparing Different Groups

When performing the bovine elastin based EnzChek[®] elastase activity reaction without any specific NE inhibitor, various endogenous proteases including NE are present in saliva such as macrophage-derived elastase and gelatinase may be contributing to the digestion of the elastin-BODIPY[®] compound releasing its fluorescence (Mecham et al., 1997). The measurement of this protease activity is demonstrated in **Fig 4.3.4.3.1**. HC saliva had significantly lower levels of protease activity than BDa after 95 min at 25°C ($p<0.02$).

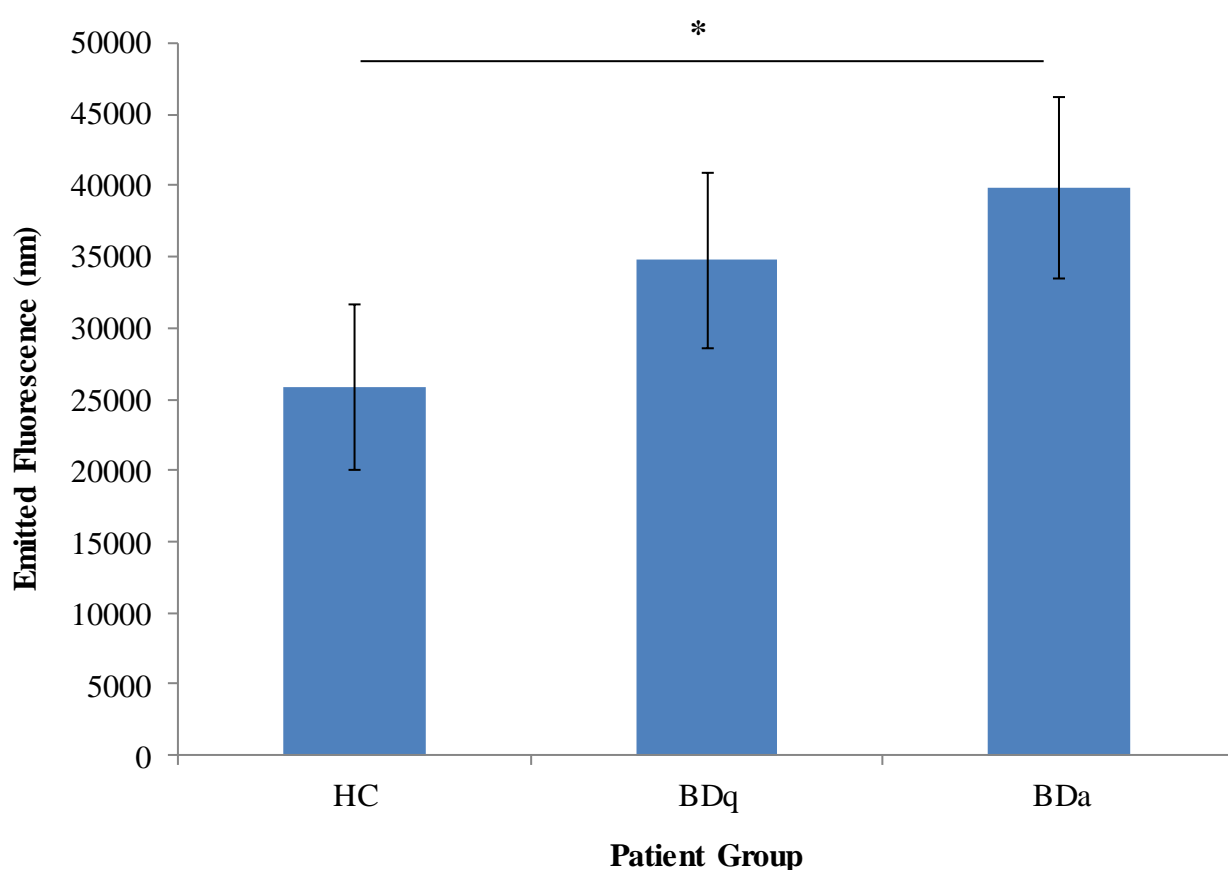


Figure 4.3.4.3.1. Comparing the Total Protease Activity in Saliva from Each Patient Group. Total protein concentration in each saliva sample was quantified and normalised to 1.5 mg/ml. Healthy Control (HC), n=30. BDquiet (q, no oral ulcers), n=33. BDactive (a, oral ulcer present), n=29. Significance determined using the Parametric Independent Sample T Test, * $p<0.02$.

4.3.4.4. Specific NE Activity in Saliva: Comparing Groups

The same EnzChek[®] assay was run again to determine the total protease activity in saliva, but this time duplicate saliva samples were simultaneously tested with 200 μ M of the chloromethyl ketone specific NE inhibitor added. This assay was completed in order to determine the definitive level of endogenous NE activity present in saliva samples from BD, RAS, and HC groups. The results can be seen in **Fig 4.3.4.4.1**. A large amount of the ketone inhibitor, 200 μ M, was necessary to inhibit more than 77 % of NE activity in all the diluted saliva samples. HC saliva, which has been shown in the ELISA to have the least amount of NE, also showed the lowest percentage (77.2 %) of NE activity. Meanwhile, 87.2 % of NE activity was inhibited in RASa saliva, the same group shown to have the highest NE levels. BDa had 82.5 %, BDq 81.5 %, and RASq 82.8 % of their NE activity inhibited. This suggests that NE is the main enzyme in saliva which is capable of digesting elastin in all patient groups.

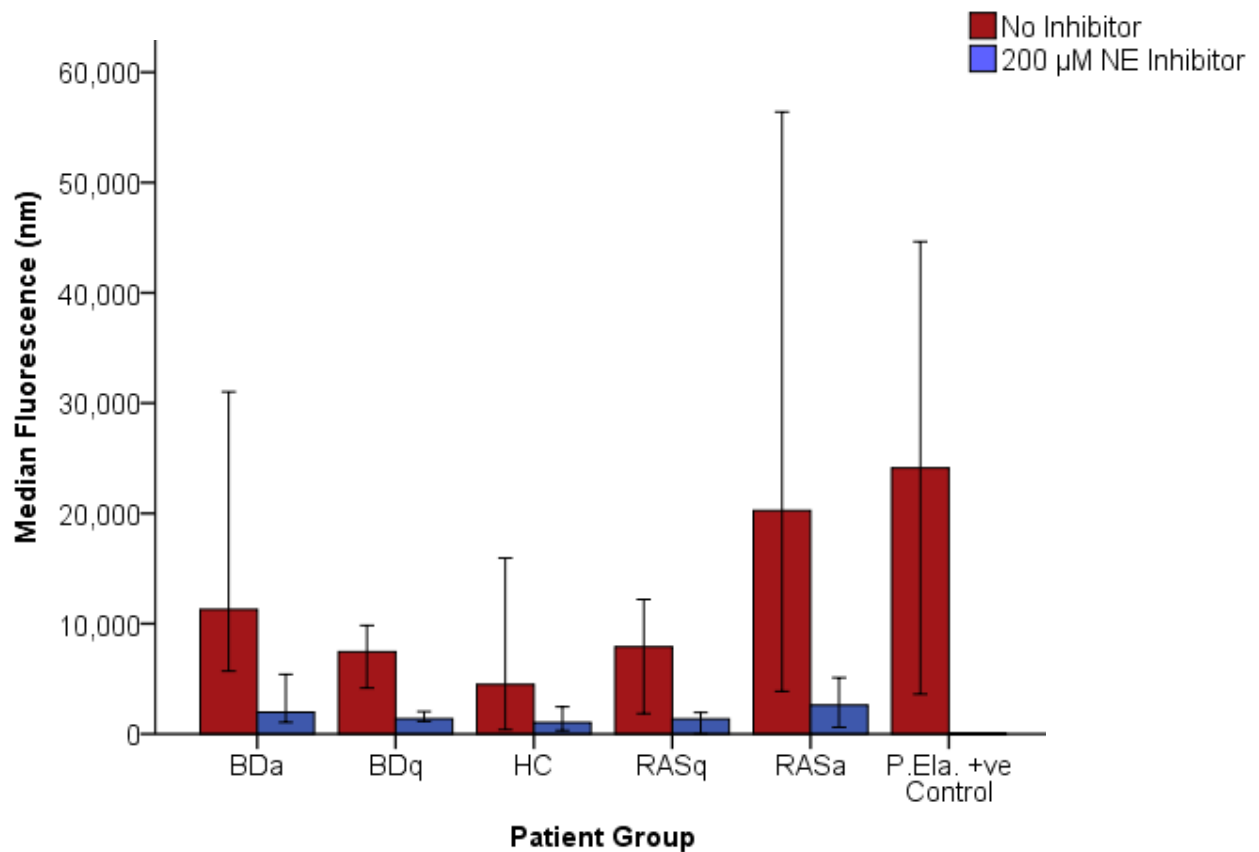


Figure 4.3.4.4.1. Specific NE Enzymatic Activity in Saliva. The total protease activity in saliva was measured by the digestion of fluorescent-conjugated elastin. A separate aliquot from the same saliva samples had 200 µM of specific NE inhibitor, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, added. NE-specific digestion of elastin was also measured in these reactions simultaneously. Saliva samples were tested from each patient group: BDa (active oral ulcer) n=7, BDq (quiet, no ulcer) n=6, HC n=8, RASq n=5, RASa n=5. The bars in red show the group median values of the total protease activity. The blue bars are the group median values for the same saliva samples with 200 µM of NE inhibitor. Error bars indicate the 95% Confidence Intervals. The reduction in elastin digestion in samples with NE inhibitor demonstrates its specific NE activity. Porcine elastase (P.Ela.) was used as a positive (+) control: its digestion of elastin was inhibited 100% by NE inhibitor.

4.3.5. Real Time Quantitative PCR

Due to the apparent SLPI depletion in saliva, the expression of SLPI mRNA in the oral cavity was investigated. Oral buccal mucosa epithelial cells were obtained from all patient groups and had their RNA extracted and transcribed to cDNA in order to determine if SLPI was up- or down-regulated during oral ulceration.

4.3.5.1. Validation and Controls

4.3.5.1.1. Bioanalyzer

After nucleic acid extraction and digestion of remaining genomic DNA, OBS RNA samples were tested for RNA quality and integrity using Applied Biosystem's Agilent Bioanalyzer before being used for any downstream assays. If RNA quality is compromised, the analysis could be unreliable.

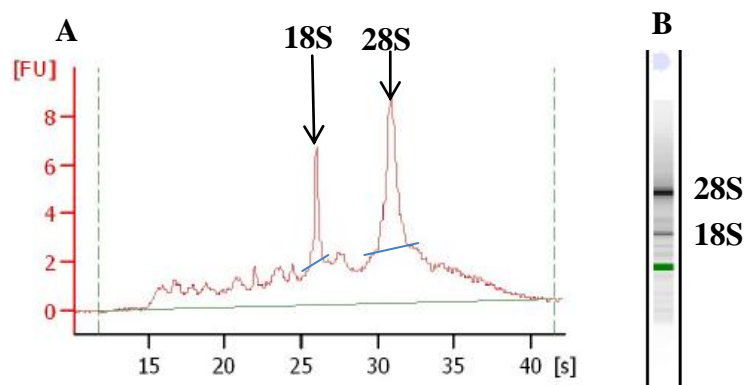


Figure 4.3.5.1.1. A and B. A: Electropherogram of an RNA OBS Sample. This graph displays a data plot of size and migration time in seconds (x-axis) as 1 µl of OBS RNA sample runs through a picoRNA chip electrophoresis matrix (Applied Biosciences) revealing the fluorescence intensity units (FU) of the RNA (y-axis). The first large peak represents the 18S rRNA; the second peak is the 28S rRNA. From the height and area of these peaks, the 18S:28S ratio, as well as the entire trace that run along the baseline, the quantity and purity of the RNA sample can be deduced. A RNA Integrity Number (RIN) is issued to grade the RNA on a scale of quality from 1 to 10 (10 being the best). **B: Electrophoresis of an RNA OBS Sample.** A second visualisation of the same RNA sample running through the electrophoresis matrix. The two dominant rRNA bands can be detected.

4.3.5.1.2. Validated Reference Genes for Oral Epithelial Cells

Roche's 19-human reference gene panel and qBasePlus software (Biogazelle, Belgium) was used to analyse the most stable mRNA reference genes in oral epithelial cells in HC, RAS (with or without an ulcer), and BD (with or without an ulcer). The results revealed that the most stable gene was GAPDH followed by the next top five: PPIA, PGK1, ACTB, YWHAZ, and 18S rRNA (see **Chapter 6**). PPIA was subsequently used as a reference gene to normalise the data for the SLPI mRNA qPCR assays.

4.3.5.1.3. Inhibition Assessment Assay

An inhibition assay was conducted in order to test if there were PCR inhibitors in the final cDNA samples. PCR inhibitors are substances that can cause inefficiency of a qPCR reaction introduced during the multi-process procedure of preparing DNA or RNA (cDNA) for gene amplification. These substances include reagents that are introduced during the sample collection, such as heparin, or extraction procedure such as ethanol or phenol, or can be remnants of the RT enzyme. The spud assay, originally designed by Nolan, *et al.*, (Nolan et al., 2006b) was conducted on different dilutions of OBS cDNA in order to see if spiking a known concentration of a foreign target (in this case, a 101 bp potato sequence) can be fully amplified or “recovered” in the presence of the cDNA sample. The quantification cycle (Cq) of the amplified target in the cDNA sample (for example, if the target amplifies at Cq 26) is compared with its amplification in water (the control) which gives Cq 24. Then it can be determined that the cDNA sample is slightly inhibited because it takes 2 more PCR cycles to amplify its target. This equates to a 7.7 % deviation from the water control. In **Figure 4.3.5.1.3.1**, the % of Cq deviation from the water control is calculated for each sample. A deviation of 0% signifies no inhibition in the sample while a deviation of 100% is total inhibition of amplification. As seen in **Figure 4.3.5.1.3.1**, when every neat sample was tested, the deviation percentage was above 9 %. This will alter the true quantification of the target gene. Any inhibitors in the OBS cDNA, which is most likely due to the presence of RT enzyme, are overcome with a 1:10 dilution of cDNA. Therefore, cDNA must be diluted before commencing a qPCR reaction in order to ensure that the gene targets are amplified efficiently and accurately.

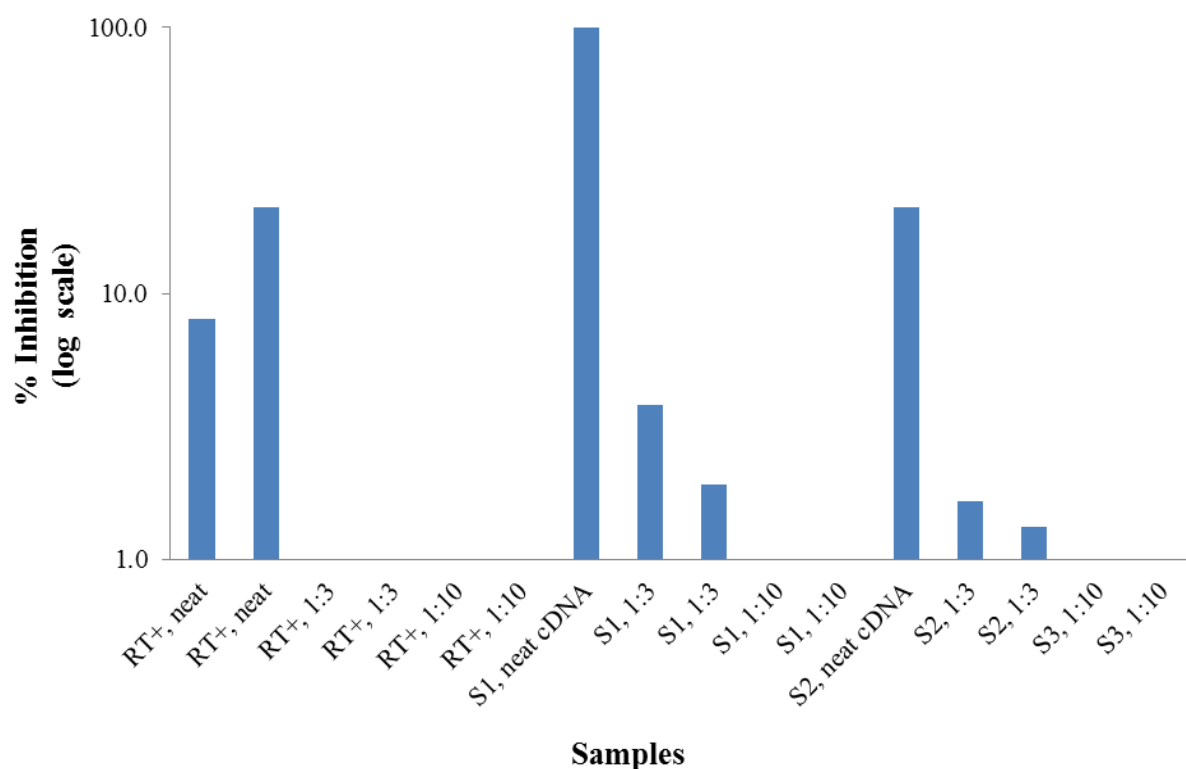


Figure 4.3.5.1.3.1. Spud Inhibition Assay Results: cDNA Must Be Diluted Before Use in qPCR Assays. Several samples and their dilutions had the spud amplicon added to them before (spud) amplification. These samples included: two oral buccal swab (OBS) cDNA samples, S1=cDNA Sample 1, S2=cDNA Sample 2, S3=cDNA Sample 3, and a water sample that underwent reverse transcription (RT+). They were added to the spud qPCR assay as a neat sample, diluted 1:3 or 1:10. A separate water sample spiked with the same spud amplicon was run as the non-inhibited control. The bars represent the difference between the quantitation cycle (Cq) of the non-inhibited water control and the spiked cDNA samples. Neat (un-diluted) samples in the qPCR assays ranged from 8% to 100% inhibited. Therefore, diluting cDNA samples is required before being analysed using qPCR.

4.3.5.2. SLPI mRNA Expression Levels in OBS

SLPI mRNA expression was measured in adult human oral buccal mucosa epithelial cells from HCs, BD and RAS patients during and in between presentation of an ulcer. There were several statistical differences in the OBS mRNA expression between groups (**Fig. 4.3.5.2.1**). The largest significant difference was between HC and BDq ($p \leq 0.001$) which suggests that even when there is no ulcer present, the oral mucosal epithelial cells of BD patients have upregulated SLPI mRNA. RASq also showed significantly upregulated SLPI mRNA ($p < 0.05$) compared to HC. BDa patients' buccal swabs that were taken away from their ulcerated area (BDa, NU) showed that the SLPI mRNA was significantly higher than HC ($p < 0.05$). The BDa, NU SLPI expression was also higher than the samples taken directly from the ulcer (BDa, U) which is probably due to necrotic tissue and therefore a lack of active metabolism and SLPI production. The non-ulcerated epithelial cells in the oral cavity may compensate for the ulcerated tissue.

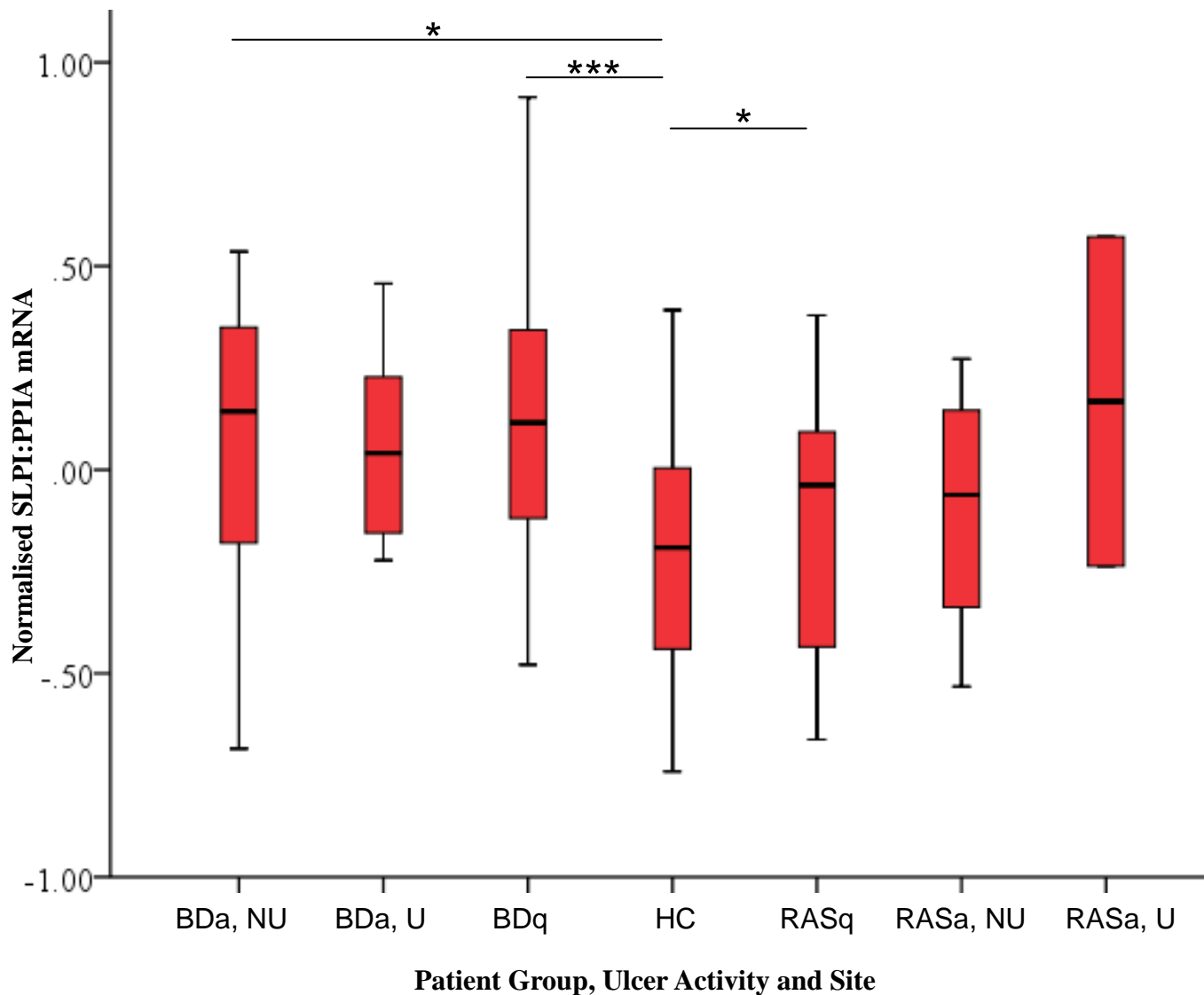


Figure 4.3.5.2.1. OBS SLPI mRNA Expression Levels in Different Patient Groups and Ulcer Sites. SLPI Cq values were first normalised using the qBasePlus Biogazelle software against the Cq values for the PPIA reference gene. The black centre bar in each box of the plot represents the median SLPI mRNA expression measured for each group. The red box represents 50% of the values in the group and the extended bars indicate the extent of maximum and minimum values therefore encompassing the range. **HC** (healthy control), **RAS** (recurrent aphthous stomatitis), **BD** (Behçet's disease). **q**, indicates mouth quiet/no ulcers. **a**, refers specifically to active mouth ulcer(s) regardless of other systemic symptoms. **NU** = swab taken from the buccal mucosa away from the oral ulcer. **U** = swab taken directly from the ulcer. BDa NU n=20, BDa U n=6, BDq n=27, HC n=16, RASq n=11, RASa NU n=4, RASa U n=2. Data assessed by PAWS/SPSS V18: Mann Whitney U, non-parametric statistical test comparing median values of two independent groups (2-tailed). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.3.6. Oral Biopsies Histology

4.3.6.1. NE and SLPI in Oral Ulcer Buccal Mucosa

Expression of NE and SLPI was investigated in biopsies from BD patients and in control tissue using immunofluorescence. The available clinical data and oral pathologist's (Prof K. Piper) conclusions following histological examination are summarised in **Table 4.3.6.1.1**.

All biopsies were excised from the left or right buccal mucosa. The outermost edge of the biopsies exhibited some non-specific antibody staining due to the antibodies becoming trapped underneath the biopsy edge. Therefore fluorescent-stained biopsy edges were ignored.

As seen in **Fig 4.3.6.1.1**, the oral ulcer from the control (Bx1) had minimal NE staining although there was some light clustered staining just below the basal cell layer of the epithelium and a small area of the submucosa. As expected SLPI expression was observed in the epithelium. Also in Bx1, SLPI staining appears in some connective tissues within the submucosa with no positive SLPI staining in the lamina propria. Bx2 showed a minimal amount of epithelium in sections and SLPI was not detected. However, there was extensive NE staining throughout the lamina propria and deep submucosa (**Figure 4.3.6.1.2**).

In Bx3, a minor saliva gland and duct was unintentionally excised from the buccal mucosa but was instrumental in demonstrating SLPI-positive staining in the acini (**Figure 4.3.6.1.3**). The inner lining of the duct adjacent to the gland also stained positive for SLPI. The epithelium also shows SLPI although the biopsy had partially folded in on itself upon cutting and mounting onto the slide. NE was not prominent in the tissue.

Bx4 had extensive distribution of NE within the lamina propria (**Fig. 4.3.6.1.4**). SLPI but not NE was found in the epithelium. SLPI was also detected in the submucosa. It had a stringy, pattern which may be SLPI staining along fibres of the connective tissue.

Patient Biopsy Number	Patient Group	Sex	Age	Ulcer Description	Ulcer(s) Duration Prior to Excision	Histological Conclusion by Pathologist
Bx1	Non-BD (control)	M	59	Hyperplasia, ulcerative changes	n/a	hyperkeratosis and widespread non-specific ulceration
Bx2	BD	F	28	Minor and herpetiform	10 days	Non-specific ulceration consistent with BD diagnosis
Bx3	BD	F	30	n/a	n/a	Non-specific ulceration, non-specific perivascular cuffing as seen in BD
Bx4	BD	M	48	Major	6 days	Non-specific ulceration

Table 4.3.6.1.1. Patient Descriptions and Biopsy Details. One oral buccal epithelium biopsy was donated by a non-BD patient who was undergoing clinical assessment for oral ulcers with hyperplasia. Biopsies from three Behçet's disease (BD) patients, 1 male (M), and 2 female (F), were also collected. A description of their ulcer and its duration was recorded whenever available, but this was not always the case (n/a). An oral medicine pathologist, Professor Kim Piper, provided a histology report on a portion of the same biopsy.

SEE BIOPSY FIGURE 4.3.6.1.1.

Fig. 4.3.6.1.1. Oral Ulcer Biopsy from Non-BD Patient. Bx1. Clinical investigation for hyperplasia & ulcerative changes. Identified as Bx1. DAPI DNA (blue), NE (red), SLPI (green). Arrows indicate main areas of positive staining for NE and SLPI. 10X magnification. Scale bar 100 μ m.

SEE BIOPSY FIGURE 4.3.6.1.2.

Fig. 4.3.6.1.2. Oral Ulcer Biopsy from BD Patient. Bx2. Minor and herpetiform ulcers for 10 days. DAPI DNA (blue), NE (red), SLPI (green). Arrows indicate main areas of positive staining for NE and SLPI. Giemsa-stained biopsy included for orientation. 10X magnification. Scale bar 100 μ m.

SEE BIOPSY FIGURE 4.3.6.1.3.

Fig. 4.3.6.1.3. Oral Ulcer Biopsy from BD Patient. Bx3. DAPI DNA (blue), NE (red), SLPI (green). Arrows indicate main areas of positive staining for SLPI. Enlarged image shows a minor saliva gland and duct from the buccal mucosa which stained positive for SLPI. Giemsa-stained biopsy was included for orientation. 10X magnification. Scale bar 100 μ m.

SEE BIOPSY FIGURE 4.3.6.1.4.

Fig. 4.3.6.1.4. Oral Ulcer Biopsy from BD Patient. Bx4. Patient had a major ulcer for 6 days prior to excision. DAPI DNA (blue), NE (red), SLPI (green). Arrows indicate main areas of positive staining. Giemsa and H&E stained biopsy sections were included for orientation. 10X magnification. Scale bar 100 μ m.

4.4. Discussion

4.4.1. Salivary NE Levels in the Presence and Absence of Oral Ulcers

The salivary NE concentration in BD and RAS patients with oral ulcers was significantly higher than the same patient groups without oral ulcers. This eludes that both groups have the innate neutrophil response that was expected during a wound (Dovi et al., 2004). The ELISA assay also revealed the normal, HC baseline of NE in the mouth. The concentration ratio of NE:SLPI was 2:1 based on the HC group median. RASq had a similar ratio closer to 1:1. When an ulcer was present, the RASa saliva revealed an 8:1 ratio. Both BDa and BDq resulted in exactly the same 6:1 ratio with NE dominating over SLPI. In order for a wound to heal effectively, the presence of neutrophils and extracellular NE needs to dissipate. The trend in our patient groups showed that NE levels were reduced in RASq, but not BDq patients.

It is with concerned interest that higher levels of NE were detected in BD patients' saliva without the presence of an ulcer. Neutrophils and extracellular NE can perpetuate inflammation, delay appropriate healing, and because of its NE's proteolytic nature can destabilise various proteins of the ECM (Szpadarska et al., 2003, Yager and Nwomeh, 1999). Circulating neutrophils and increased NE have been previously implicated in the systemic chronic inflammatory response in BD (Deger et al., 1995, Eksioglu-Demiralp et al., 2001), but until this present study, was not linked to the extracellular presence of the protease in saliva and its potential damage to the oral mucosa.

Quantifying a higher NE level in BD compared to RAS patients suggests that when an oral ulcer is absent, RAS patients have a more subdued innate immune "resting" state than BD patients. RASa patients may resolve the influx of neutrophils more efficiently than BDa. However, RASq sample size representation was quite low in these assays (n=10), and additional measurements are necessary to confirm significant differences. Therefore, while it can be suggested that NE plays a central to the oral pathology seen in BD, its role in RAS ulcers is still uncertain.

4.4.2. SLPI mRNA in Oral Buccal Epithelial Cells and Protein Level Discrepancy

Before the results of the SLPI protein levels quantified by ELISA can be explored in detail, it is necessary to discuss the oral epithelial cells which contribute to producing local SLPI mRNA. Recently, two real time qPCR-based studies were able to collect and access SLPI mRNA expression in healthy oral buccal epithelium using cytobrushes

(Taborda et al., 2012) and punch biopsies (Nittayananta et al., 2013a). These healthy controls were used to compare HIV-seropositive patients SLPI expression. Unfortunately, both studies also failed to validate their reference gene for normalisation. Without the validation that the reference gene is stable in all samples and sample treatments, the expression results are inaccurate and therefore misinterpreted. Our study was the first to measure SLPI mRNA in the oral buccal epithelium from HC as well as BD and RAS patients during quiet and active episodes of ulceration alongside a validated, stable reference gene. (See Chapter 6 for more details on oral buccal epithelial cells reference gene validation).

For all the individuals tested, there was no genetic deficiency associated with oral SLPI since mRNA was able to be quantified in each sample. Interestingly, even when oral ulcers were absent, the buccal mucosal epithelial cells of BD patients showed highly upregulated SLPI mRNA. This is probably a response to the chronic presence of inflammatory markers, NE, and other unknown stimulants. Unexpectedly, RAS patients without ulcers also showed significantly higher SLPI mRNA expression compared to HC. While this coincides with the RASq SLPI protein level being higher than HC, both groups shared similar NE levels in their saliva. Therefore, salivary NE may not be solely responsible for stimulating the increased epithelial SLPI mRNA expression in RAS patients.

Additional RASa and BDa OBS samples are necessary to deduce significant differences between patients with active ulcers, especially at the direct site of the ulcer. These samples were difficult to obtain due to the pain of placing a brush onto the sensitive surface of the ulcer which would frequently aggravate the wound. Also, after testing several of these direct-ulcer RNA samples on the Bioanalyser, several showed low quality and had to be excluded from the study at this checkpoint. This created a limitation of the study.

A previous study on sputum found that SLPI concentrations measured by ELISA were higher in COPD patients when compared to HC non-smokers (Tsoumakidou et al., 2010). In our study, it was predicted that the salivary SLPI concentrations would be higher in individuals with oral ulcers due to the fact that it is part of the inflammatory response to a wound (Angelov et al., 2004). However, surprisingly, when comparing the different patient groups, SLPI protein levels decreased significantly in BD patients whether or not they had ulcers present. This contradicts the mRNA results which indicate that oral

epithelial cells from BD patients (with or without ulcers present) have significantly increased expression of SLPI. Presumably this means that the cells are producing high quantities of SLPI protein, although this was not detected with the ELISA. The success of the translational process of SLPI (mRNA to protein) is unknown in this instance, however, our data would suggest that 1) the production, folding, or exocytosis of the SLPI protein is insufficient during ulceration or 2) SLPI may be “used-up” or bound (complexed) with NE (or other proteases and microbes) thereby causing degradation of the SLPI protein or masking of the SLPI antibody epitope. Unfortunately, the epitope information for the antibody used in the present study is not available from the manufacturer in order to confirm this theory. However, we believe the latter to be the most likely cause of the mRNA-protein discrepancy due to the inverse relationship seen in NE and SLPI ELISA results.

Similar discrepancies between SLPI mRNA and SLPI protein measurements have been shown in other studies (van Wetering et al., 2000) (Saitoh et al., 2001) (Jana et al., 2005). Van Wetering, *et al*, presumed their juxtaposing results were due to an increase in cell-associated as opposed to cell-secreted SLPI after stimulation of bronchial epithelial cells by NE in culture. Saitoh, *et al*, interpreted similar results as a delay in exocytosis of SLPI from epithelial cells since the cells’ first contact with NE may release a large initial amount of stored SLPI. It has been shown that a short *in vitro* incubation of *ex vivo* human tracheal submucosal serous gland cells with NE can result in increased exocytosis of SLPI protein (Maizieres et al., 1998), but cells could be too slow to replace the protein (Jana et al., 2005). From our findings thus far, we can contribute that SLPI is produced and released into the oral cavity but that it is met with high levels of NE and α 1AT. The levels of SLPI produced do not seem to be sufficient to regulate the consistently high concentrations of NE that are seen in BD. This is assuming that the levels of these three proteins measured in HCs are the levels that are required for homeostasis to be maintained in the oral mucosa.

A study by Sallenave, *et al*, showed that SLPI mRNA was up regulated when a lung epithelial cell line was stimulated with IL-1 β and TNF (Sallenave et al., 1994). SLPI mRNA has also been shown to be induced *in vitro* by NE (Jin et al., 1997). Therefore, the high up-regulation of SLPI mRNA could be directly due to the high level of NE in the mouth.

In another experiment, Saitoh, *et al*, combined α 1AT and NE into SLPI-producing epithelial cell cultures (Saitoh et al., 2001) resulting in the SLPI mRNA being significantly reduced compared to a culture with only NE added (without α 1AT anti-protease). Therefore, if there is a relatively large amount of competitive inhibitor (α 1AT) already present in an NE-laden environment, the induction of SLPI mRNA production could be reduced. In our experiments, salivary α 1AT had higher protein concentrations than NE and SLPI. Therefore, it could be deduced that even if SLPI mRNA is upregulated, it still may not translate to sufficient levels of protein to regulate NE since an over-abundance of α 1AT is present in the oral environment.

4.4.3. The Inverse Relationship of NE and SLPI Protein

A direct relationship between NE and α 1AT levels was apparent in our ELISA measurements, however, there was an unexpected inverse relationship between NE and SLPI. As previously discussed, this phenomenon could be due to SLPI being degraded and inactivated, bound to other proteases such as CatG, or inhibiting microbes – all of which could cause masking of the compatible configuration necessary for antibody detection. The SLPI depletion could also be because not enough is being produced to meet the demands despite SLPI mRNA upregulation and/or once the protein is released from the cells, it is overwhelmed by increased NE.

The inverse relationship between SLPI and NE protein was also previously described in oral fluid samples by Cox, *et al*, in 2006. They found increased NE and decreased SLPI in gingival crevicular fluid and diluted whole mouth saliva (Cox et al., 2006). Our ELISA data concurs with this excess of NE over SLPI in the oral environment based on protein concentration ratios. The low levels of measurable SLPI may be a consequence of the high levels of NE if the proteins have complexed and cannot be detected. This theory is supported by similar assays conducted by Saitoh, *et al*, in 2001 in which they suspected the epitope of their commercial SLPI monoclonal antibody coincided with the protein's active inhibitory site (Saitoh et al., 2001).

The type of physical binding of SLPI with NE has been demonstrated by Koizumi, *et al*, in 2008, but they only worked with half of the SLPI protein – the C-terminal domain that binds to NE (Koizumi et al., 2008). In theory, its binding with NE could block the SLPI detection antibody, but this clearly does not occur 100% of the time since our

experiments have shown that we are able to measure over 850 ng/ml of SLPI by ELISA as well as visualise it in the saliva and OBS as one clear protein band in Western blots at the 12 kDa expected size. The question remains whether our ELISA is detecting either complexed or unbound SLPI, or both.

Exploring SLPI's response to a stimulant, a study conducted by Grobmyer, *et al*, in 2000 showed fluctuating SLPI measurements in plasma over 48 hrs after intravenous injection of LPS into healthy controls. They used the same SLPI ELISA from R&D Systems that we employed. After baseline measurements, SLPI levels seemed to rapidly decrease - the lowest occurring 3 hrs post-LPS challenge. Then, there was a rapid increase above baseline concentrations peaking at 12 hrs after which the levels descended and returned closer to baseline levels by 48 hrs. Both high and low LPS dosages experienced the same fluctuations although the higher dosage increased the range of maximum and minimum SLPI levels (Grobmyer et al., 2000b). In our experiment, patients with active ulcers were shown to have decreased SLPI, similar to the initial exposure of LPS. Based on patient group analysis, BD patients never seemed to replenish or recover their salivary SLPI levels signifying that a constant stimulant is present. Most insightful is when no ulcer is present, BD patients still have what seems to be depleted SLPI while RAS patients have high concentrations. This would suggest that BD patients endure a constant stimulant regardless of oral ulceration while RAS patients do not and are able to recover an immunogenic equilibrium.

Grobmyer, *et al*, speculated that there could be an issue with the detection of SLPI by ELISA. They suggested that SLPI could be degraded by proteases (which were upregulated by the LPS) or that SLPI was bound to an unknown receptor. This binding may also mask the antigen-antibody recognition making it "invisible" to the ELISA (Grobmyer et al., 2000b). This technical misrepresentation was also demonstrated by two similar ELISA results. The first was testing an in-house ELISA developed by Westin, *et al*, in which two antibodies to human SLPI (raised in mouse and sheep in-house) were used to conduct a sandwich ELISA. A fixed concentration of recombinant human SLPI was mixed with varying NE concentrations in PBS and measured on the SLPI ELISA. This basic analysis revealed that when more than double the amount of NE was introduced to rSLPI, the SLPI concentration was reduced nearly two-fold (Westin et al., 1999a). It is worth noting that the same result occurred with Cathepsin G but not with chymase. SLPI binds both of these but perhaps at different sites allowing the ELISA antibody to detect SLPI at one site but not another. The second example, a study

conducted by Sullivan, *et al*, sought to validate the R&D Systems' ELISA measurements of SLPI. Using purified proteins, they found that increasing NE > 100:1 (NE:SLPI stoichiometric molar ratios) steadily decreased the successful detection of rSLPI (Sullivan et al., 2008). The authors of these experiments theorised that this apparent SLPI depletion may be due to the NE:SLPI complex configuration masking the SLPI antibody detection or an over-abundance of NE clouding the SLPI antibody detection. Westin, *et al*, suggested that the ELISA may be giving "false low values". In our circumstances, a heterogeneous saliva sample already contains both SLPI and NE, therefore the total SLPI concentration may already be masked at a baseline reading since endogenous NE concentration always seems to be higher even in healthy controls.

4.4.4. High Levels of Alpha-1 Anti-Trypsin in Saliva

It was surprising to see that the α 1AT in saliva, even in HCs, was at such high concentrations since several other studies reported lower concentrations of salivary α 1AT. Pederson, *et al*, measured only 5 - 240 ng/ml of α 1AT by ELISA in healthy control unstimulated, whole saliva (Pederson et al., 1995). In a study by Cox, *et al*, the α 1AT values in whole saliva from severe chronic periodontitis patients reported a vast range of 1,272 - 16,748 ng/ml (Cox et al., 2006) but not concentrations above 1×10^6 ng/ml as our study had. In both previous studies, the saliva samples were diluted 1:2 with an equal amount of buffer. In order to confirm that our patients' saliva samples were being measured by the α 1AT ELISA accurately and to clarify mixed reports, stored serum samples from the patients and HC donors were diluted in PBS at 1:100, 1:10,000, and 1:100,000 and measured since serum is known to carry high quantities of α 1AT. The 1:100,000 diluted serum levels showed that HC had a mean of 1.97×10^6 ng/ml which was within previously reported ranges. For instance, Mornex, *et al*, revealed that α 1AT in normal serum had a mean of 2.03×10^6 ng/ml (range 1.24 - 3.40×10^6 ng/ml) (Mornex et al., 1986). Another study found that the α 1AT content in plasma from healthy, normal donors was between 1.5×10^6 - 3.5×10^6 ng/ml when measured by immunodiffusion (Dickens and Lomas, 2011) while others reported a similar range (in serum and plasma) from 1.2×10^6 - 2×10^6 ng/ml by standard nephelometry (Sun and Yang, 2004). The confirmation of high quantities of α 1AT measured in serum both in previous studies and using our ELISA method helped to validate the high concentrations measured in our patients' saliva. It is proposed that the saliva samples used in Cox, *et al*, and Pederson, *et al*, studies were not sufficiently diluted before being assessed by ELISA. Therefore, in

our study, the predominant anti-protease concentration in saliva was α 1AT and not SLPI as was hypothesised.

The results from our investigations showed there was a strong association between salivary levels of α 1AT and NE. At this time it is not clear whether an increase of NE in saliva is actually stimulating the increased recruitment of α 1AT from circulating serum but this could be inferred. If the α 1AT:NE are irreversibly complexed, it is unknown whether this interferes with their concentration measurements using ELISA. In future experiments, it is vital to determine if the high NE levels measured by ELISA are actually already complexed with α 1AT. If this is the case, the risk of ECM being digested by NE would be depleted. The western blots we conducted showing multiple NE protein bands suggest that antibodies could simultaneously target NE and NE bound to other proteins. A study by Pederson, *et al*, (1995) was able to measure the NE: α 1AT complex in saliva using an ELISA assay with α 1AT as the capture antibody followed by NE primary detection antibody (Pederson et al., 1995). It would appear that the antibody epitopes they used were capable of attaching to complexed proteins. When NE is complexed with α 1AT, it is functionally inactive (Carrell et al., 1982, Gavrin et al., 2012b, Korkmaz et al., 2008b). Again, this information is important for understanding NE's activity and potential tissue damage in the oral cavity.

From our evidence, we propose that SLPI and α 1AT levels are influenced by NE and not necessarily by the sole presence of oral ulcers since BD patients without oral ulcers still have high NE. It is a cyclic relationship in which NE will perpetuate increased chemotaxis of additional neutrophils and NE inhibitors. In 1988, J. Travis described NE as a stimulus for SLPI in the lung (Travis, 1988). But perhaps SLPI levels are also influenced by the surprisingly high concentration of α 1AT in the saliva. SLPI and α 1AT are beneficial competitors in the sense that they have a specific affinity to inhibit the same potentially damaging NE enzyme. Both can be found internally in neutrophils (Amulic et al., 2012, Clemmensen et al., 2011, Paakko et al., 1996) presumably protecting the cell itself from unintentional proteolytic damage. Both are also found in gingival crevicular fluid, although α 1AT is serum-derived and SLPI is thought to be from the gingival junctional epithelium (Nakamura-Minami 2003). However, since SLPI is also produced locally by oral epithelial cells and the salivary gland acinar cells, it was expected to be the main salivary inhibitor for NE, however, α 1AT which is produced in liver cells and transferred to the serum, was more abundant. The fact that α 1AT was found in such high quantities in saliva yet the NE present was still able to actively degrade elastin was a

surprising find since as a serpin, α 1AT, is thought to irreversibly bind and inactivate NE (Korkmaz et al., 2008b). However, even with the surplus of α 1AT, the NE was still active, even in HC. This occurrence was also found by Vignola, *et al*, (1998) in the sputum samples of asthmatic and chronic bronchitis patients (Vignola et al., 1998). They proposed that α 1AT could be inactivated by reactive oxygen species, such as superoxide anions and hydrogen peroxide, or loses function when bound to neutrophil metalloproteinases, such as NE, and collagenase (Michaelis et al., 1990).

The reactive centre loop on an α 1AT molecule, which acts as an attractive cleavage site for NE, has a methionine residue which is susceptible to this oxidation (Matheson et al., 1979). Not only are phagocytic cells such as neutrophils and macrophages capable of generating oxidizing agents but so are bacteria at the site of infection. Logistically, it would be counterproductive for NE to be immediately inhibited by α 1AT since this would allow bacteria to overwhelm the host's tissues. It may be the case, as Carrell, *et al*, proposed, that at the periphery of an infected site, the α 1AT may better contain the protease activity, but at the centre of infection, the various oxidizing species from neutrophils, macrophages, and bacteria downgrade the α 1AT inhibitor (Carrell et al., 1982, Schonberg et al., 2012).

Our enzymatic assays and antibody-binding stages of the ELISA's have been carried out under neutral pH conditions. Patient saliva was diluted at least 1:20 and 1:200 in PBS, respectively, when undergoing analysis due to its high total protein content. In saliva, α 1AT was at least 5 times the concentration of NE in HC, BDq, RASq, and BDa patient groups. If α 1AT, a serpin-type inhibitor, binds and inactivates NE irrevocably, then this would most likely diminish the NE enzyme activity. However, the NE activity measured in BDa was still significantly higher compared to HC. This suggests that the salivary α 1AT is not fully active in order to regulate salivary NE. This may further be explained by the dampened activity of α 1AT in the slightly acidic pH of human saliva (Reibetanz et al., 2012, Schonberg et al., 2012) which normally can range from a pH of 5.3 – 7.8 (Humphrey and Williamson, 2001a). If neutrophils and extracellular NE are present especially in the absence of an oral ulcer (as seen in BDq), reactive oxygen species could further contribute to lowering saliva pH rendering α 1AT inactive thus creating another protease-inhibitor imbalance.

4.4.5. Target Proteins Identified by Western Blots

The polyclonal goat SLPI antibody was useful in identifying one, clear SLPI protein band in various oral specimens just above the 12 kDa protein ladder. The rSLPI may be a slightly less cationic than the native protein as it ran a fraction slower during the electrophoresis process resulting in a band slightly higher than the samples. Saliva samples that were used on SLPI westerns were also targeted with NE antibodies. The expected 29.5 kDa NE protein band was detected. The protein content in these western blots coincided with the quantity of the saliva sample measured on the ELISA. Purification columns (DNeasy kit, Qiagen) with an acetone protein precipitation were used to isolate protein from whole blood and OBS samples, but the fluid portion of saliva was not filtered or purified further, therefore could have resulted in the two closely separated NE bands.

It was expected that employing the use of a purified, monoclonal NE-HRP conjugated antibody would result in one band at the 29.5 kDa mark. Surprisingly, there were additional protein bands identified in the NE westerns. The three bands, with different degrees of intensity, showed up at approximately 12, 31, and 52 kDa in the majority of saliva samples. In the NE-HRP western blots, the lower band hovering just above 12 kDa in several saliva samples could be a component of NE. The suppliers of the purified, native NE derived from human blood neutrophils (Abcam, UK) suggested that the lower bands were probably due to some NE degradation. Finally, all samples including saliva, isolated protein from blood, and ulcerated oral epithelium revealed the large band just above 52 kDa.

Isolated protein from the oral keratinocyte culture supernatant also revealed the 52 kDa band. This sample was meant to be a negative control for the 29.5 kDa NE, which was absent, but it was not anticipated that a larger band would be identified. It is suspected that this larger band was a residual NE: α 1AT complex from the serum used in the keratinocyte culture media. As there is no further NE production released from these cells and α 1AT is generally found in higher quantities in serum than NE, the NE was bound by excess α 1AT and detected on the western blot. Therefore, it is a strong possibility that the reoccurring large band in all the other patient samples is the NE: α 1AT protein complex.

An inhibitor-enzyme complex of SLPI and NE has been previously been described (Koizumi et al., 2008, Sullivan et al., 2008, Morrison et al., 1990, Chan et al., 2003). If SLPI bound NE and formed a strong complex in saliva which was tolerant of 70°C and 50

mM DTT reduction, it was expected that this complex could be detected on the western blot around 41.2 kDa (11.7 kDa SLPI + 29.5 kDa NE). This was not the case in any of the samples or the combination of purified proteins. It could be that this complex was disrupted since it is believed to be reversible (Gauthier et al., 1982) during protein reduction (DTT or heat treatment) or sample processing (freeze-thaw).

Saitoh, *et al*, proposed their antibody may also have been recognising some SLPI proteolytic products (Saitoh 2001), but we did not find proof of these smaller protein fragments on our SLPI western blots. The smaller fragments lead to an interesting observation also previously reported by Cox, *et al*, (Cox et al.) in which cleavage products of SLPI less than 12 kDa were detected by western blots. Degradation of SLPI has also been shown by Weldon, *et al*, using increasing concentrations of purified NE over SLPI (Weldon et al., 2009). Cysteine proteases such as Cathepsins B, L, and S in the mouth as well as chymase, a serine protease from mast cells (Belkowski et al., 2009), can cleave and inactivate SLPI (Cox et al., 2006) (Taggart et al., 2001). However, it has not been reported if cleavage can occur once SLPI is complexed with a protease. If SLPI was shown to be cleaved in our samples, presumably it would affect the function of the inhibitor and result in NE being deregulated which would be detrimental to the oral tissues. Low molecular weight SLPI cleavage products or fragments were not observed on any of our western blots, therefore we can infer that from our results this pathological mechanism is not occurring in our patients groups. However, the use of native, non-reducing gels may show a better representation if the SLPI is being degraded.

4.4.6. Salivary Proteases and NE Activity

As a proof of principle, the ability of recombinant SLPI to inhibit purified human NE was demonstrated at a 1:3 ratio of NE:SLPI. An *in vitro* study previously reported that recombinant SLPI was able to inhibit free NE on a 1:1 molar ratio (Morrison et al., 1990). However, another study combined non-recombinant native SLPI isolated from lung epithelial cells with purified NE and measured SLPI by ELISA. They found that 25 times more SLPI was necessary to inhibit NE (Saitoh et al., 2001). In this experiment and our own, a larger amount of SLPI was required to inhibit NE activity. Interestingly, our ELISA results showed that salivary SLPI was consistently found in lower concentrations in comparison to NE even in HC individuals. Perhaps this represents its working ratio which according to the ELISA averaged 2:1 (NE:SLPI). After all, it would be detrimental to inhibit all NE as it has an important role in innate immunity.

The same recombinant SLPI from R&D systems was also tested by Pilette, *et al.*, on PMA-stimulated blood neutrophil cultures, resulting in 90% inhibition of 0.05 μ M NE activity when mixed with 2.5 μ M of rSLPI (Pilette et al., 2003). Similarly in our experiment, 69% of 0.385 μ M NE was inhibited by 1.08 μ M rSLPI.

To confirm that the NE measured in saliva was biologically active, a NE-specific inhibitors (*N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone) was added to BD, RAS, and HC patients' saliva samples. The exogenous inhibitor revealed that all groups had highly active NE in their saliva causing elastin cleavage. The two highest activities were associated with the two groups having an oral ulcer: RASa and BDa. The NE activity corresponded to the NE concentrations measured with total protein being normalised for each sample. This outcome was not altogether unexpected since the presence of an ulcer would usually stimulate neutrophil recruitment in order to control any bacteria invading the wound. However, the BDq group also had a higher NE activity than HCs. Individuals with BD have been described as having hyperactive neutrophils in their blood circulation (Verity et al., 2003, Matsumura and Mizushima, 1975). Our data adds to this observation that active salivary NE is also present. Furthermore, high levels of enzymatically active concentrations of NE in BD saliva could contribute to recurrent mucosal damage and ulceration associated with this disorder. The question whether increased NE in saliva can be regulated effectively by SLPI is therefore of important clinical relevance in these patients.

The same chloromethyl ketone inhibitor, at 200 μ M, was previously used to show > 90% inhibition of 0.05 μ M of degranulated NE derived from phorbol-myristate-acetate (PMA) stimulated blood neutrophil culture (Pilette et al., 2003). A similar buffer at pH 7.5 and reaction temperature at 37°C was used for our first assay with the ketone inhibitor.

Similarly to α 1AT, it is speculated that a portion of native SLPI could be deactivated *in vivo* by reactive oxygen species. Oxidation has been shown to occur at the same protease-inhibiting site – the methionine residue (Korkmaz et al., 2008b). This could be a naturally occurring balance of enzyme and inhibitor. Unfortunately, native SLPI from each patient's saliva sample could not be isolated in this study, therefore limiting investigation of the function and activity of native, human salivary SLPI (only the recombinant form is available commercially). It may be possible in the future to do this using chromatography and HPLC purification in order to use it in assays as they have done from sputum samples

(Hochstrasser et al., 1981), but at the moment, due to the large volume of saliva that is required for purification and the cost entailed, this was not a logistical consideration.

In saliva from all patient groups tested, NE can retain its activity in saliva when handled according to the conditions observed for the current study which included: storing on ice at clinic, centrifuging at 4°C, aliquoting and freezing aliquots on dry ice at the lab bench, then transferring these immediately for long term storage to a -80°C freezer until use.

4.4.6. Distribution of NE in Ulcerated Oral Buccal Epithelium

4.4.6.1. Distribution of NE

NE was found within the lamina propria and submucosa in the ulcer biopsies although there was extensive distribution in two biopsies from BD patients. Despite their similar NE expression indicating neutrophil infiltration, these patients had very different clinical features. One patient was experiencing an acute episode of activity. Along with the mouth ulcers, they also had genital ulcers, folliculitis, and CNS issues. Interestingly, this person was not on any medication. Quite differently, another BD patient had oral ulceration, was considered to be non-relapsed, and was medicated with azathioprine and betnesol mouthwash. The biopsy from the third BD patient showed minimal NE expression yet this patient was relapsed with joint and folliculitis manifestations and was reported to use triple mouthwash for the ulceration but was not on any systemic medication for BD. From this data, NE expression in ulcerated tissues does not reflect BD disease activity.

In all four biopsies, NE expression was never directly detected in the epithelium or above the stratum basale layer. This was surprising as it was expected that neutrophils would inundate the area of an ulcer, but it is likely that other areas around the ulcer, especially if the epithelium was compromised or perforated, provided the chemotactic factors necessary to recruit neutrophils. A few CD3⁺ T-lymphocytes were identified within the epithelial layers of all biopsies except for BD Bx2 since there was no epithelium present in the section (data not shown). Although CD3⁺ cells were in relatively small numbers in the epithelium, a large congregation of CD3⁺ cells were evident just below the stratum basale border inside the lamina propria in the control biopsy and more extensively in the same two BD biopsies with extensive NE infiltration described above. The appearance of CD3⁺ T-cells which are part of the adaptive immune system is compatible with the fact that the mucosal tissue from these patients had been ulcerated for 6 and 10 days. The age

of the ulcer could also be the reason why NE expression was not found in the intact epithelium since neutrophils are usually the first to migrate to a wound and usually subside after a few days when the adaptive immune system would dominate.

4.4.6.2. SLPI in Ulcerated Mucosa

SLPI was identified in the epithelium of the buccal mucosa in two biopsies from BD patients and in the control tissue. SLPI expression was evident throughout the epithelial cell layer, but did not frequently present below the margin of the stratum basale into the lamina propria. This staining is comparable to oral mucosal staining seen in HC biopsies (Wen et al., 2011) and confirms its expression in oral buccal epithelial cells. SLPI was difficult to detect in one of the BD biopsies because there was a very limited amount of epithelium in the section. However, it was fortuitous that in this biopsy a minor saliva gland and duct from within the buccal mucosa was excised in the biopsy specimen. Strong positive SLPI staining was observed all along the inner lining of the acini in the gland and duct cells similarly seen in Wahl, *et al*, (Wahl et al., 1997b). This provided evidence that saliva glands produce SLPI in BD patients with oral ulcers, even though their salivary SLPI levels are low. NE was not found around or within the gland, however CD3+ cells were scattered along these structures (data not shown for CD3).

In the control and one non-relapsed BD patient biopsies, SLPI also appeared to be evident lining the major vessels and some connective tissue fibres. The origin of SLPI's presence is probably serum-derived.

SLPI was difficult to stain at first since fixation with 100% ice-cold methanol on these tissues did not allow the SLPI epitope to be detected by polyclonal or monoclonal SLPI antibody. It was not until fixing with 4% PFA for 10 min and combining this with the use of a mouse monoclonal SLPI antibody that positive identification of SLPI within the epithelial cells became apparent.

Note that α 1AT was targeted on the biopsies but due to time constraints and the necessary optimisation involved with this particular antibody, α 1AT staining in these biopsies was inconclusive (data not included).

4.4.7. Conclusion

NE was found in significantly high concentrations in saliva from RAS and BD patients with oral ulcers compared to HC. The NE enzymatic activity was directly proportional to the concentration of NE in saliva. Importantly, the NE concentration was also elevated in individuals with BD even in the absence of ulceration. Due to its increased presence, NE could play an important role in the cause of oral mucosal damage and recurrent ulceration in BD.

Both NE and SLPI have anti-microbial properties and help to protect the oral mucosa against invading oral microbes. It was hypothesised that SLPI concentration would increase alongside an increase of NE during oral ulceration, but this was not the case. Instead what we found was an inverse relationship in which a higher NE concentration in saliva meant a lower SLPI concentration. Interestingly, SLPI mRNA expression was significantly up-regulated in BD oral mucosal epithelial cells with or without ulceration. It would seem that SLPI is inadequately supplied in saliva and may not be able to regulate high concentrations of NE. This in turn recruits various other inflammatory cells into the oral mucosa which continue to produce inflammatory cytokines. A chronic cycle of inflammation is created which may contribute towards direct mucosal injury leading to the manifestation of recurrent ulceration and severity of an ulcer.

The majority of BD patients (whether experiencing an active or quiet episode) are on long-term medication, such as corticosteroids and colchicine, to control inflammatory exacerbations. However, colchicine, which has been shown to reduce neutrophil chemotaxis (Matsumura and Mizushima, 1975), did not reduce the high level of salivary NE in BD patients without ulcers. Therefore, despite being on colchicine, neutrophils must still gain access to the oral cavity in large quantities since the presence of NE was increased. This reinforces the need for local inhibition of increased NE.

Application of topical recombinant SLPI has been shown to reduce inflammation, elastase activity, and the time it takes for a wound to heal (Ashcroft et al., 2000). Incorporating rSLPI in a mouthwash for individuals with oral ulcers may reduce the severity and healing time. For individuals with a systemic disorder, like BD, in which NE and pro-inflammatory mediators are constantly up-regulated, a topical mouthwash may prevent the chronic reappearance of painful oral ulcers. McElvaney, *et al*, was able to show that cystic fibrosis patients who used aerosolised rSLPI had decreased NE in their lungs

(McElvaney et al., 1993). In 2009, Li, *et al*, proved that a low cost, high yield version of biologically active recombinant SLPI could be produced using *Pichia pastoris* yeast (Li et al., 2009) providing a more practical method of in-house preparation and supply for research.

Also, a critical investigation of the native SLPI human antigen-antibody epitope combinations need to be identified in order to definitively analyse the levels of free and/or unbound SLPI protein in human specimens such as plasma and saliva. This will not only help us understand the mechanism of SLPI's inhibitory properties, but also help to discover its true concentration as this is especially important in monitoring rSLPI-based medical treatments.

Chapter 5:

NETosis in

Behçet's Disease

5.1. Introduction

5.1.1. NETosis: The Discovery of an Alternative Type of Death in Neutrophils

A histology textbook published in 1995 describes neutrophil membrane protrusions (Junqueira et al., 1995). About the same time, Takei, *et al*, reported observing an atypical cell death when human neutrophils were activated with phorbol 12-myristate 13-acetate (PMA) (Takei et al., 1996). In standard culture media (RPMI 1640 with 10% FCS) and common incubation conditions (37°C, 5% CO₂, 95% humidity), the addition of PMA into an isolated population of neutrophils produced a unique and unusual cell death involving the formation of vacuoles and the nucleus morphing into a spherical shape causing it to “spread out.” After 3 to 4 hrs, the cell ruptured spilling its contents into the extracellular space. Then in 2004, Volker Brinkmann and his colleagues at Max Planck Institute in Berlin, published their experiments describing a similar neutrophil-specific cell death entailing the externalization of particular cellular constituents which they proved had bactericidal capabilities (Brinkmann et al., 2004). They named the ruptured cells’ filaments neutrophil extracellular traps, or NETs, and this new type of programmed cell suicide became known as NETosis (Steinberg and Grinstein, 2007).

To date, many scientists have expanded the portfolio of information on NETs even though the exact mechanism is still unknown. However, Brinkmann, *et al*, were the first to describe some of the key elements required for neutrophils to undergo NETosis: neutrophil elastase (NE), myeloperoxidase (MPO), reactive oxygen species (ROS) and the main constituents of the NETs being DNA histones (Brinkmann et al., 2004). Brinkmann, *et al*, used PMA to induce neutrophils, but also included the biologically relevant stimulants IL-8 and bacterial LPS. Importantly, NETosis was found to be an active process requiring the cell to remain functional up until the stage at which NETs are released and the cell dies (Brinkmann et al., 2004). They proved that activated, live neutrophils were releasing NETs by using exclusion dyes, such as sytox[®] green, which only penetrate the cell membrane when integrity is lost. They also found that only live, motile cells could produce NETs.

NETosis can be differentiated from the other cell death pathways – apoptosis and necrosis. Apoptotic cells condense and fragment their chromatin without the disintegration of the nuclear envelope (Fuchs et al., 2007). Also, NETosing cells do not express phosphatidylserine phospholipid, the receptor for the Annexin V marker, which

occurs in apoptosis or when the cell membrane is compromised. Fuchs, *et al*, demonstrated the morphology of neutrophils that underwent necrosis when exposed to bacterial toxin in order to help distinguish this type of cell death from NETosis. They found that during necrosis, the cells retained their nuclear envelope and a clear separation of granules and nuclear components was seen (Fuchs et al., 2007). Takei, *et al*, also showed that when stimulated with PMA, none of the neutrophils showed chromatin agglutination which is a feature of necrosis (Takei et al., 1996).

It has been shown that NETs are released in response to microbial infection (Brinkmann et al., 2004). More specifically, it is thought that this pathway is a response to an overwhelming influx of infectious organisms such as in severe infection or sepsis (Ma and Kubes, 2008). The launched web of decondensed chromatin has been observed by scanning electron microscopy (Brinkmann et al., 2004). Cellular proteins such as NE and MPO bind and biologically inhibit pathogens (Brinkmann et al., 2004, Fuchs, 2007 #3576, Fuchs et al., 2007). To date, sixteen pathogens have been tested and proven to induce a NET response (Brinkmann and Zychlinsky, 2012). These include some common bacterial species, such as *Staphylococcus aureus* and *Escherichia coli*, and the yeast, *Candida albicans*. Even *Aspergillus* spp. fungal hyphae, which are too large to phagocytose, have been eliminated by NET filaments (Bianchi et al., 2009).

Most of the early work on NETs involved *in vitro* stimulation of neutrophils with PMA, LPS, IL-8, or co-incubations with pathogens (Brinkmann et al., 2004, Fuchs et al., 2007). More recently, NETs occurring *in vivo* have been investigated in order to be morphologically described. Histological sections from a rabbit infected with shigellosis were able to show extracellular NE and histone fibres (Brinkmann et al., 2004). Another *in vivo* animal model with *Klebsiella pneumonia* determined that wild-type mice could produce NETs while NE-knockout mice could not (Papayannopoulos et al., 2010).

5.1.2. The Role of DNA, Neutrophil Elastase and Myeloperoxidase in NETs

As the main constituents of the fibrous chromatin NET scaffold, NE, a potent serine protease, and MPO, a peroxidase enzyme which makes hypochlorous acid (HOCl) in the presence of hydrogen peroxide (H₂O₂) and chloride anion, have the potential to actively penetrate microbial cell membranes and cleave bacterial virulence factors (Logters et al., 2009, Papayannopoulos and Zychlinsky, 2009). In addition to their enzymatic activity,

both NE and MPO are cationic proteins that can inhibit bacterial growth by binding to the pathogen's negatively-charged surface. MPO, a glycosylated 146 kDa dimer protein, is stored along with NE in the azurophil granules in abundance. MPO can also be found in lower concentrations in monocytes but not tissue macrophages (Malle et al., 2007). These two enzymes are usually released into a neutrophil's phagolysosome to internally digest opsonised pathogens in a controlled, enclosed environment.

MPO is itself non-oxidative, but it catalyses the oxidation of halides (such as chloride anion, Cl^-) using H_2O_2 to create hypohalous acids (mainly the unstable oxidizing agent HOCl) and other highly reactive antimicrobial products. HOCl can then go on to participate in oxidation and halogenation reactions (Malle et al., 2007). Upstream of this cascade, located at the neutrophil's phagosomal and cell membrane, nicotinamide adenine dinucleotide phosphate (NADPH) supplies the electrons needed to reduce oxygen to superoxide anions. The superoxide simultaneously reduces and oxidizes creating H_2O_2 which is the substrate for the MPO-catalysed reaction. Individuals with complete MPO-deficiency cannot make NETs (Metzler et al., 2011) which highlights the importance of MPO in the NETosis pathway. Also, people suffering from the severe immunodeficiency disorder Chronic Granulomatous Disease (CGD), have a NADPH oxidase gene defect and therefore cannot produce ROS. Their neutrophils also do not form NETs (Fuchs et al., 2007). However, by restoring NADPH oxidase via gene therapy or by adding exogenous H_2O_2 , they can regain the ability to make NETs. Therefore, the molecular pathway involving NADPH, ROS, and MPO are important contributors to enable NETosis to take place.

The role that NE plays in NETosis has become one of the definitive characteristics of this pathway. Previous experiments have shown that NE relocates from the azurophil granule and enters the nuclear space (Papayannopoulos et al., 2010). Here it is thought to digest the histones, mainly targeting H4, thereby unravelling the supercoiled DNA chromatin structure (Papayannopoulos et al., 2010). Histones are cationic proteins which help to condense DNA in the nucleus. Two of each of the core histone proteins (H2A, H2B, H3, and H4) are grouped together creating a cylindrical structure. Short sections of dsDNA wrap around these core histones creating individual nucleosomes. The DNA strands are secured in place by H1, a linker histone. Many millions of nucleosomes are then supercoiled to form the chromosome. Histones themselves have also been shown to have potent anti-microbial effects by promoting cell lysis (Hirsch, 1958, Kawasaki and

Iwamuro, 2008, Kim et al., 1996). Globular domains for H2A-H2B-DNA have been detected sitting among long stretches of NET fibres (Brinkmann et al., 2004) suggesting their involvement in the capturing and inactivating of bacteria.

It is thought that the azurophil granules remain intact during NETosis. They do not enter the nuclear region, but individually, two of its major components (NE and MPO) have been identified co-localising with intracellular DNA (Papayannopoulos et al., 2010), however, MPO has been shown to transport separately into the nucleus at a later time point than NE enhancing the decondensation process (Papayannopoulos et al., 2010). Experiments conducted by Papayannopoulos, *et al*, ruled out proteinase 3 and cathepsin G, two proteases that are also located in the azurophil granule, in the breakdown of neutrophil DNA (Papayannopoulos et al., 2010). It is not known by which mechanism NE and MPO are able to be selectively transported. Once NETs are released, additional proteases have been identified in the structure such as Cathepsin G and bactericidal permeability-increasing protein (BPI) from azurophils, lactoferrin and gelatinase from specific and tertiary granules, respectively (Brinkmann et al., 2004). No membranes are seen surrounding the chromatin fibres. Proteins not present in NETs included the granule membrane protein CD63, actin, and tubulin (Brinkmann et al., 2004).

5.1.3. Endogenous Inhibitors of NETs

SLPI and α 1AT can both be found circulating in human plasma (Grobmyer et al., 2000a). Their concentrations have been reported to increase in individuals suffering from chronic inflammation, such as in COPD (Hollander et al., 2007). Hepatocytes are the main producers of α 1AT while mucosal epithelial cells and salivary gland acinar cells generate SLPI (Ohlsson et al., 1984). Neutrophils also produce and harbour these protease inhibitors intracellularly. SLPI can be found in specific granules, also referred to as secondary or peroxidase-negative granules, while α 1AT has been located in the same azurophil granule as NE and MPO (Mason et al., 1991). More recently, α 1AT was also found in multiple neutrophil granules including, the specific granules, gelatinase granules, and secretory vesicles (Clemmensen et al., 2011). SLPI and α 1AT's involvement in the NETosis pathway have only been reported as extracellular components once NETs are expelled, however they have not yet been characterised intracellularly during this cell death process.

5.1.4. NETosis, or ETosis, in Other Granulocytes

Thus far limited to granulocytes, the active release of DNA and cell contents with an antimicrobial purpose is unique. The process of inducing Extracellular Traps (ETs) in eosinophils (Yousefi et al., 2008) and mast cells (von Kockritz-Blickwede et al., 2008) has been reported. Yousefi, *et al*, showed that IL-5 or IFN- γ primed *ex vivo* human eosinophils to discharge DNA and cationic proteins *in vitro* when stimulated with LPS and complement factor 5a (C5a) and to a lesser degree eotaxin. Interestingly, there was no ETosis without priming. In the same study, intestinal biopsies from five individuals with Crohn's disease were also found to have mast cell and eosinophil IL-5 expression as well as evidence of eosinophilic extracellular DNA throughout the tissue. Bacterial lipopolysaccharide (LPS) has also been shown to stimulate mast cells to release their contents, however this was only tested in mouse-derived or human cell line mast cells as opposed to isolated *ex vivo* human mast cells (von Kockritz-Blickwede et al., 2008).

5.1.5. The Pathology of Neutrophil Extracellular Traps

It is thought that NETs evolved to contain and fight the spread of infection. However, they have also been identified in non-infectious conditions such as in preeclampsia (Gupta et al., 2005) and psoriasis (Skrzeczynska-Moncznik et al., 2012). NETs may be partly responsible for the direct destruction of inflamed tissues due to the enzymes and ROS released. It is well documented that one of the main NET constituents, NE, is capable of cleaving various host proteins that construct the extracellular matrix such as elastin which leads to the degradation of connective tissue. The NE in NETs could therefore contribute to tissue damage.

NETs can also instigate and/or perpetuate an inflammatory response. It is thought that they may contribute to the pathogenesis seen in autoimmune diseases with chronic inflammation such as in rheumatoid arthritis (RA). Recently, Khandpur, *et al*, found NETs in the skin and synovial fluid from RA patients. In addition, RA serum and synovial fluid could induce NETs in HC neutrophils *in vitro* (Khandpur et al., 2013). The autoimmune diseases Systemic Lupus Erythematosus (SLE) and small-vessel vasculitis (SVV) have elevated levels of anti-neutrophilic cytoplasmic antibodies (ANCA) which have been implicated in the pathogenesis. The most common ANCA autoantibodies target Proteinase 3 (PR3) and MPO, but in SLE, antibodies against DNA and histones are also targeted (Branzk and Papayannopoulos, 2013, Tsokos, 2011). Therefore, NETs are thought to be an additional extracellular source of the target antigens. Also, ANCA

perpetuate the inflammatory cycle by activating neutrophil respiratory burst and production of ROS by binding to surface-expressed PR3 and MPO. The on-going inflammation causes capillary damage. Kessenbrock, *et al*, found that TNF- α primed neutrophils incubated with isolated ANCA-IgG from SVV patients could induce NET formation (Kessenbrock *et al.*, 2009). Similarly, autoimmune complexes from SLE patients could stimulate NET production in HC neutrophils (Garcia-Romo *et al.*, 2011). In both SVV and SLE, NETs were also found deposited in kidney biopsies (Villanueva *et al.*, 2011, Kessenbrock *et al.*, 2009). NETs may be implicated in the pathogenesis of BD vasculitis. Although auto-antibodies have not been fully described in BD to date (Direskeneli, 2006), it is still a possibility that one exists. There have not yet been any investigations on the occurrence of NETs in BD patients in their vasculature, mucosa, tissues, or organs.

It has been shown that there are several ways in which neutrophils can be recruited into the oral cavity. GCF is thought to be a major route for neutrophils into the mouth (Cox *et al.*, 2006). We have previously seen in **section 4.3.6.1** that neither neutrophils nor released NE were identified in intact epithelial oral tissue above the basal cell line, but were recruited in large quantities when the tissue was compromised by ulceration. These macroscopic ulcers as well as common microscopic abrasions that occur in the mouth are an obvious route for blood-derived neutrophils to gain access through the mucosal lining. However, the question still remains why a higher concentration of NE, which is mainly reserved for intracellular digestion of bacteria, is found in the saliva of BD patients who suffer episodic ulceration regardless of the presence of an ulcer. This prompted an investigation to find out if BD patients harboured circulating neutrophils undergoing a high frequency of NETosis, and therefore extracellular NE, which could potentially reach the oral cavity propagating or initiating ulcers.

5.1.6. Aims

Our objectives of the experiments were to investigate:

1. Whether *ex vivo* neutrophils from BD patients undergo a higher occurrence of NETosis than HC. This will be determined by observing the convergence of NE and MPO with intracellular DNA during NETosis as well as the quantification of extracellular DNA.
2. Upon inducing NETosis, whether neutrophils from BD patients and HCs undergo a similar process involving NE and MPO.
3. To determine if NE is actively contributing to cellular NETosis by blocking it with synthetic NE inhibitor. Also, to see if this type of inhibition can down-regulate the occurrence of NETosis.
4. And finally, where endogenous neutrophil-derived SLPI and α 1AT inhibitors are located during NETosis and if they co-localise with NE intracellularly and/or extracellularly.

5.2. Materials and Methods

The final experimental design for investigating NETs *in vitro* was arrived at following several initial optimisation protocols for isolating and culturing neutrophils, and for imaging neutrophils undergoing NETosis.

5.2.1. Optimisation Experiments

5.2.1.1. Initial Sytox[®] Green Experiments and Culturing Neutrophils on Poly-D-Lysine Coated Coverslips

Extracellular DNA from isolated neutrophils were successfully detected using sytox[®] green dye while in the culture wells (**Figure 5.2.1.1.1**). Sytox[®] green (Life Technologies, UK) does not penetrate intact cellular membranes only binding to extracellular DNA that is released upon cell death. While some extracellular neutrophil DNA remained within the immediate boundary of the cell's outer membrane creating a cylindrical shape (**Figure 5.2.1.1.1**, arrow), most of the DNA stretched across large areas attaching to and encompassing neighbouring cells' DNA. These strands of DNA are thought to be the hallmark of NETs (Fuchs et al., 2007) and therefore observation of these web structures signifies that cells are undergoing NETosis. When imaged, the exposure time had to be increased so that both the cells and their extended DNA strands could be visualised in a single image.

Initial experiments also incorporated testing different concentrations of poly-D-lysine used coat glass coverslips to improve neutrophil attachment for imaging. This methodology has been reported in several papers (Garcia-Romo et al., 2011). Glass coverslips were coated with 0.001% (or 10 µg/ml) of poly-D-lysine with a high molecular weight (150-300 kDa) to encourage cell attachment. Fibrous strands of extracellular DNA were evident in the unstimulated and positive control PMA-stimulated neutrophil cultures using a 1:15,000 sytox green dilution (final concentration in each well was 0.33 µM) as described by Pappayannopoulos, *et al*'s. **Figure 5.2.1.1.2** shows neutrophils from a relapsed BD patient undergoing NETosis. Neutrophils from other BD patients (data not shown), and surprisingly also from HC subjects (data not shown), revealed similar reactions and therefore this unexpected result alerted us to the fact that something else was happening in our neutrophil cultures that was inducing NET formation. Following several lines of questioning we came to the conclusion that it was perhaps the poly-D-lysine that was causing NETosis. Therefore, we hypothesised that NET formation would increase with increasing Poly-D-lysine concentration hence the same number of HC

neutrophils were seeded onto coverslips coated with increasing concentrations of Poly-D-lysine. Indeed, the number of neutrophils NET forming increased with increasing Poly-D-lysine concentration. Subsequently, coating glass coverslips with poly-D-lysine was aborted. Instead, sterile non-coated glass coverslips were used instead for all cultures processed for immunocytochemistry while 24-well polystyrene, flat bottom culture plates (Nunc, VWR, UK) were used for in-well sytox[®] green imaging.

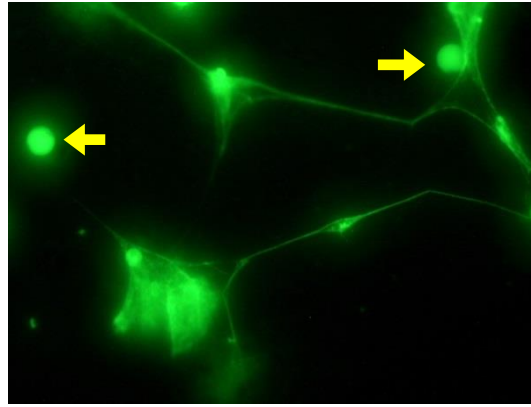


Figure 5.2.1.1.1. Initial Culturing Method: In-Well Imaging of Unstimulated Neutrophils from a BD Non-Relapsed Patient Cultured onto 0.001% Poly-D-Lysine Coated Glass Coverslips. Cells were stained with sytox[®] green (1:15,000 dilution) and imaging was completed 1 hr after cells were distributed in culture. In-well imaging completed on inverted Nikon fluorescent microscope (GFP channel). Characteristic net-like configurations of extracellular DNA were observed. Some cells remained cylindrical (arrow). X40 magnification.

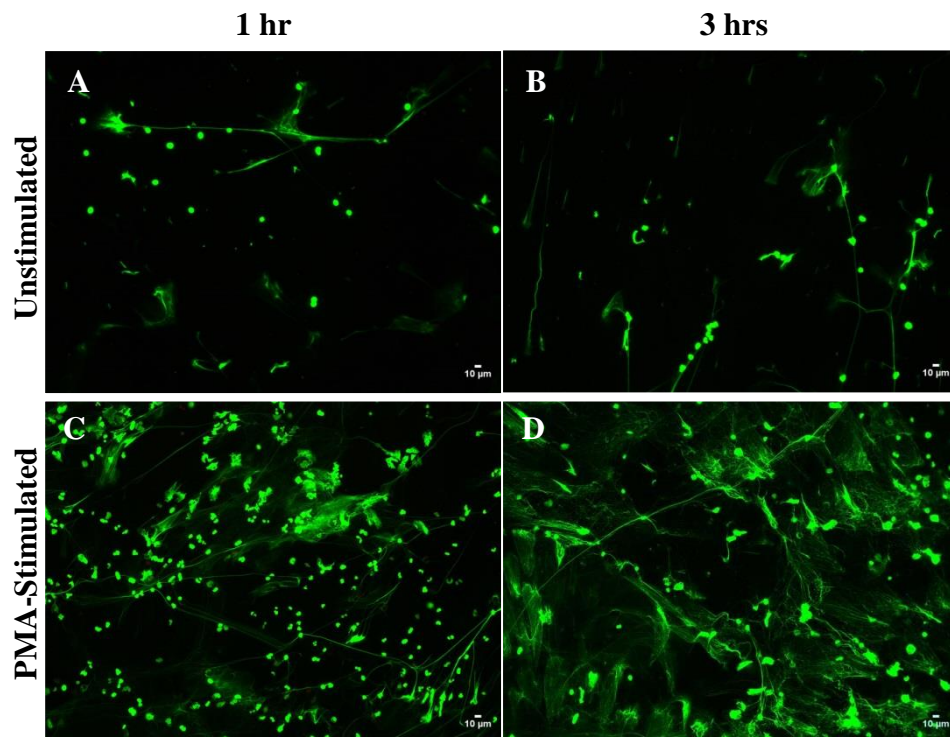


Figure 5.2.1.1.2. Neutrophils from Relapsed BD Patient Cultured onto 0.001% Poly-D-Lysine Coated Glass Coverslips and Mounted. Cells were either treated for 1 hr with (C) or without (A) PMA. Another duplicate culture was incubated for 3 hrs with (D) or without (B) PMA. At the specified time points, cells were stained in culture with sytox green and then coverslips delicately removed and inverted onto mounting medium. Images taken on an Epifluorescent Leica microscope, X20 magnification. Bar indicates 10 μm.

5.2.2. Isolation of Neutrophils from Whole Blood for Investigation of NETs *In Vitro*

Neutrophil isolation was based upon the protocol described by Brinkmann, *et al*, (Brinkmann et al., 2004) using Histopaque 1119 and percoll gradients. Therefore, Histopaque 1119 (Sigma, UK) was equilibrated to RT at least 2 hrs before use. Several days prior to conducting the procedure, the 13 mm, thickness number 1.5 non-coated borosilicate glass coverslips (VWR, UK) were sterilized by autoclave. 1X and 10X PBS was also prepared and autoclaved.

All blood work was carried out in a Class II Safety Cabinet. Histopaque 1119 (Sigma, UK) (6 ml) was added to sterile 15 ml falcon tubes. Then carefully, 5 ml of whole blood was layered on top and tubes were centrifuged at RT for 20 min at 800 x g without braking. Using a sterile pasteur pipette, the plasma was removed from the top layer (**Figure 5.2.2.1, A**), transferred in a separate tube, and placed in -80°C freezer for future experiments. The next layer consisting of PBMC's was also collected separately, washed in PBS by centrifugation (300 x g, 10 min) to create a cell pellet to which 0.5 - 1 ml of RLT lysis buffer (from RNeasy Qiagen kit, UK) was added. This was stored in the -80°C for future nucleic acid extraction.

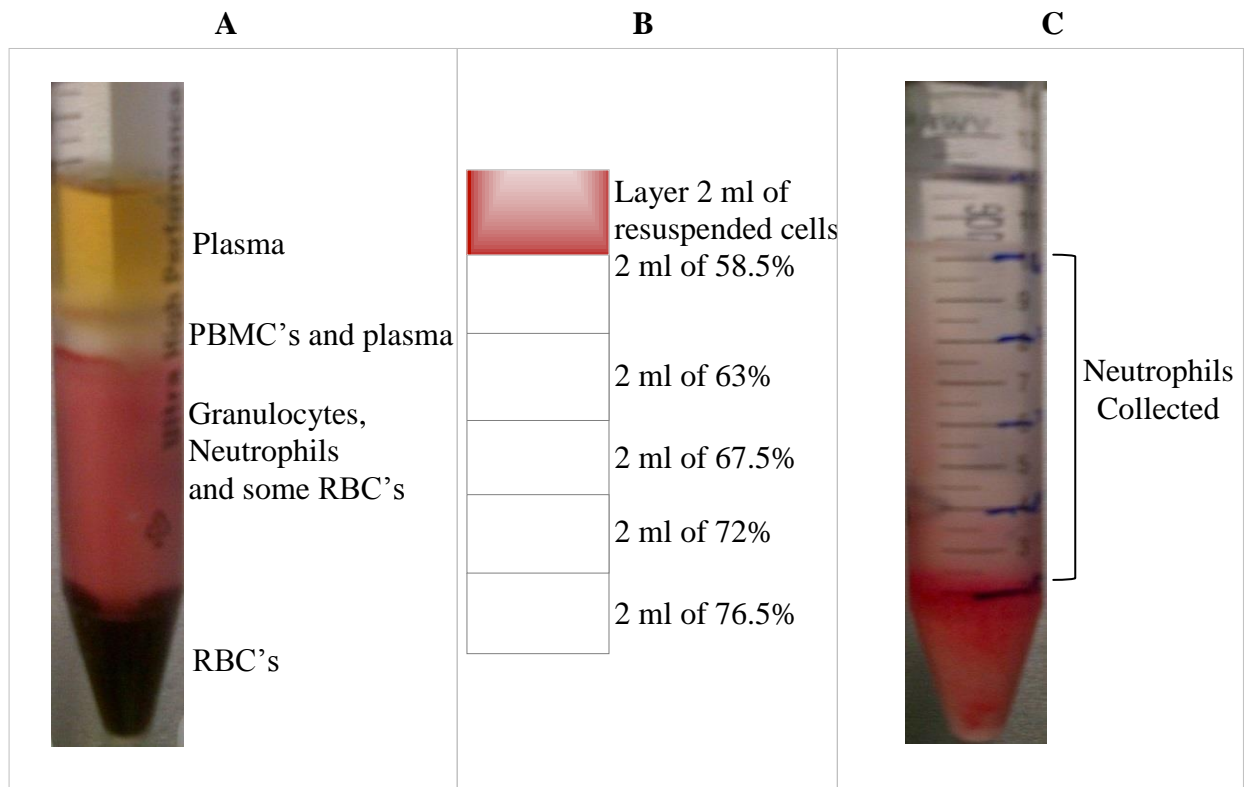


Figure 5.2.2.1. A, B, C. A. The Layers of Blood after Histopaque Separation. The top yellow plasma layer was transferred by pipette to a new tube for freezer storage. The next layer containing mainly PBMCs was also collected separately and stored. Next, the pink layer containing neutrophils and other granulocytes was carefully collected without disturbing the RBC layer and transferred into a new 15 ml tube. The RBC layer was discarded. **B. Layering the Percoll Gradients.** In a 15 ml centrifuge tube, 2 ml of the highest percoll concentration (76.5%) was added first followed by the careful layering of 2 ml of the next highest concentration. Layering was continued with all subsequent percoll preparations. Lastly, 2 ml of the resuspended granulocyte pellet added followed by centrifugation at RT for 20 min at 800 x g without braking. **C. Collecting Neutrophils within the Percoll Gradients.** Approximately 4 ml of the cloudy, white layer consisting of neutrophils was collected and transferred to a new tube. The first 2 ml of clear fluid was discarded. RBC's gathered toward the bottom layer were avoided.

The lower pink mid-section of the resulting Histopaque layers contained the granulocytes and some RBC's. This layer was carefully collected by pipette and transferred into new 15 ml Falcon tubes. The bottom dark red layer containing only RBC's was discarded. The collected granulocytes were washed with sterile 1X PBS and centrifuged for 10 minutes at 300 x g. Meanwhile, a 90% Percoll solution was prepared by mixing 18 ml of 100% Percoll with 2 ml of 10X PBS. Then with 1X PBS, several diluted solutions of percoll were prepared in separate tubes: 76.5%, 72%, 67.5%, 63%, and 58.5% (2 ml was prepared for each patient blood sample). 2 ml of each Percoll gradient was carefully layered on top of each other starting with the highest 76.5% as shown in **Figure 5.2.2.1, B.**

After cells were centrifuged, the supernatant was discarded and the cell pellet was resuspended in 2 ml of PBS. If the pellet was particularly large with abundant RBC's, then the pellet was suspended in 4 ml PBS. A maximum of 2 ml of the resuspended pellet was then carefully layered onto the top gradient, therefore if the resuspension of cells was 4 ml due to a large pellet, then 2 gradient preparations were required for each sample. This was then centrifuged for 20 min at 800 x g without braking at RT. After centrifugation, the top clear layer and most of the 58.5% layer (which contained a cloudy band of residual PBMC's) were removed (**Figure 5.2.2.1, C**). The remaining interphases were collected into a new tube avoiding the lowest layer where the RBC's congregated. Cells were washed by addition of 1X PBS and centrifuged for 10 min at 300 x g after which the supernatant was removed and the cells re-suspended in 2 ml of culture media. Culture media was prepared consisting of RPMI 1640 (Gibco/Life Technologies, UK), 10 mM HEPES (Sigma, UK), and 5% fetal calf serum (FCS) (Gibco/Life Technologies, UK). Initially, a standard 10% of FBS was added to the media and then lowered to 5% due to its reported inhibition of NET formation (Fuchs et al 2007). The cells were then counted using a haemocytometer and their viability assessed using the trypan blue exclusion test (usually > 99% viable).

5.2.3. NET Formation

Five experimental conditions were used to study NET formation *in vitro*: 1) unstimulated (non-treated) neutrophils, 2) 100 nM of PMA-stimulated neutrophils as a positive control for inducing NETs, 3) neutrophils with 5 µM of NE inhibitor (NEi, type GW311616A from Sigma, UK) for inhibiting NET formation, 4) PMA and NEi together as an additional control, and finally 5) neutrophils with 0.01 U/µl DNase I from bovine pancreas (Sigma, UK) used as a negative control to dissolve extracellular DNA released by NETs. Immunocytochemistry and the quantitative sytox[®] green experiments were run concurrently, however for ease, the experimental protocols are described separately.

5.2.3.1. Immunocytochemical Qualitative Experiments: Antibody Staining and Fluorescent Confocal Microscopy

Neutrophils were seeded into 24-well culture plates containing sterile glass coverslips at a concentration of 250,000-500,000 cells in 400 µl which would be added into each well of a for downstream immunocytochemistry purposes. One plate was prepared for each patient and each time point. The glass coverslips were added to the wells. If they floated

on the media, they were gently pushed down with sterile tweezers so that they sat at the bottom of the well. Separate working stocks of PMA (Sigma, UK), NEi, and DNase I were prepared in media. Immediately after the cells were added to the wells, 50 µl of a 45 µM NEi working stock was added to 400 µl of neutrophils in each designated NEi well (final well volume of 450 µl). Also, 25 µl of a 90 µM NEi working stock was added to 400 µl of neutrophils in the well for NEi+PMA reactions. Both reactions contained 5 µM of NEi as the final concentrations. The cells were incubated for 1 hr at 37°C in 5% CO₂ and allowed to settle. After settling, 50 µl of media was added to unstimulated cells and 50 µl of 0.09U/µl DNase I working stock added to DNase reaction wells. Also, 50 µl of 900 nM PMA working stock was added to the PMA stimulated cells while 25 µl of a 1800 nM PMA working stock was pipetted into the NEi+PMA wells. A final volume of 450 µl media gave 100 nM PMA per well. Any well where 25 µl of reagent was added, another 25 µl of media was added to bring the final volume to 450 µl. The first time point was 2 hours after the neutrophils were initially added to the well (this includes 2 hrs of NEi incubation as well) and 1 hr of stimulation by PMA or digestion by DNase. The next time point was 2 hrs after the first, so a total of 4 hours of neutrophil incubation with and without NE inhibitor and 3 hrs of PMA stimulation and DNase reaction.

The same time points were used for the sytox green and immunocytochemistry plates. My supervisor Dr Eleni Hagi-Pavli assisted with the fixing and washing step for these plates while I conducted the sytox green imaging. Using a sterile pasteur pipette, 450 µl of media was removed gently and transferred to a new, identical 24-well plate set up in order to be stored in the -20°C. Then 300 µl of 4% paraformaldehyde (PFA) (w/v) was added to each well, incubated for 15 min (in a Class II Cabinet), removed and washed with sterile 1X PBS by adding 1 ml to each well and leaving for 5 min then discarding. This wash step was repeated twice more. A final volume of 0.5 ml of PBS was added to each well, the plate lid was placed on top, and cling film or parafilm secured around the boarder. The plates were dated and kept at 4°C until used for immunofluorescent staining.

PFA-fixed neutrophils were washed once with 0.5 ml of 1X PBS followed by addition of 300 µl of blocking buffer made up of 5% goat serum (Merck Millipore, USA), the cell permeabilising agent 0.5% saponin (Sigma, UK), and 0.05% Tween-20 (Sigma, UK) diluted in PBS. They were left for 1 hour at RT. Meanwhile, the primary antibody was diluted in preparation for use according to the optimised dilutions outlined in **Table 5.2.3.1.1**. The same blocking buffer was used as the diluent for all the antibodies.

Target	Block and Permeabilise	Antibody Stains	Antibody Dilution	Isotype Control
mNE	5% goat serum 0.5% saponin 0.05% Tw-20 in PBS	1° Ab: mouse monoclonal IgG1, 0.1 mg/ml neat concentration, (HyCult #HM2174) 2° Ab: AF555 goat, anti-mouse IgG polyclonal F(ab') ₂ region, 2mg/ml (Life Technologies, UK, Cat #A-21425) (visible in red channel)	1: 400 1:2000	Mouse IgG1 (DAKO)
pNE	5% goat serum 0.5% saponin 0.05% Tw-20 in PBS	1° Ab: NE polyclonal rabbit, anti-human, whole antiserum, IgG (Abcam, UK, Cat# ab21595) 2° Ab: AF647 goat, anti-rabbit IgG polyclonal F(ab') ₂ fragment, 2mg/ml (Life Technologies, UK) (visible in far red channel)	1: 400 1:2000	Normal rabbit serum
MPO	5% goat serum 0.5% saponin 0.05% Tw-20 in PBS	1° Ab: polyclonal rabbit IgG, anti-human, (DAKO #A0398) 2° Ab: AF647 goat, anti-rabbit IgG polyclonal F(ab') ₂ fragment, 2mg/ml (Life Technologies, UK) (visible in far red channel)	1:400 1:2000	Normal rabbit serum
SLPI	5% goat serum, 1% BSA, 0.5% saponin, 0.05% Tw-20 in PBS	1° Ab: monoclonal mouse IgG1, anti-human (R&D Systems, UK, Cat# MAB1274) 2° Ab: AF555 goat, anti-mouse IgG polyclonal F(ab') ₂ region, 2mg/ml (Life Technologies, UK) (visible in red channel)	1:100 1:2000	Mouse IgG1 (DAKO)
α1AT	5% goat serum 0.5% saponin 0.05% Tw-20 in PBS	1° Ab: polyclonal rabbit, anti-human, (Novocastra #NCL-A1Ap) (Leica Microsystems, UK) 2° Ab: AF647 goat, anti-rabbit IgG polyclonal F(ab') ₂ fragment, 2mg/ml (Life Technologies, UK) (visible in far red channel)	1:500 1:2000	Normal rabbit serum
DNA	n/a	DAPI: (Sigma, UK) (visible in blue/UV channel)	Final conc: 100 ng/ml	n/a

Table 5.2.3.1.1. Optimised Target Antibody Details, Blocking and Permeabilising Buffers, and Isotype Controls for PFA-Fixed Neutrophil NETs.

Once the blocking step was complete, the buffer was removed from each well and discarded and then replaced with 250 µl of the diluted primary antibody and left to incubate overnight at 4°C. NE and SLPI or NE and MPO or NE and α1AT primaries were tested together and could be used to stain the cells as a dual reaction in one well (as they also used different secondary antibody fluorophores). Note that a polyclonal NE primary antibody was used for dual staining with SLPI. Meanwhile, monoclonal NE was coupled with MPO or α1AT since the polyclonal NE antibody was produced in the same animal as those from MPO and α1AT and therefore would not be differentiated by the secondary antibody. Isotype controls were also used in place of primary antibodies for each target to check for specificity **Table 5.2.3.1.1**. These isotypes were diluted to the same concentration as the primary antibodies. Other wells were also allocated as controls to check for secondary antibody non-specific binding whereby only the secondary antibody was added, so during the primary antibody incubation stage, these controls would have 250 µl of only the diluent/blocking buffer added. They were all incubated overnight at 4°C without disturbance.

After the primary antibody incubation, coverslips were washed with 1 ml PBS-T, for 5 min. after which PBS-T was removed and discarded. This wash step was repeated 3 times for all wells. Before the last wash, the secondary antibody was prepared. Depending on the primary species and target, the secondary antibody was either polyclonal Alexa-Fluor® 555 goat, anti-mouse or Alexa-Fluor® 647 goat, anti-rabbit (Molecular Probes/Life Technologies, UK). Both had F(ab')₂ fragments with a neat concentration of 2 mg/ml. Secondary antibodies were diluted as indicated in **Table 5.2.3.1.1** using the same blocking buffer/diluent described above. Each well containing the coverslip-bound neutrophils received 250 µl of the diluted secondary antibody and incubated for 1 hr at RT in the dark. Afterwards, each well was washed as described above.

A 100 ng/ml solution of DAPI nuclear stain (Sigma, UK) was prepared in 1X PBS and 250 µl of DAPI solution was added to each well which were incubated for 5 min. Following staining, coverslips were carefully lifted out with the help of sharp, long curved precision tweezers (Ideal-Tek 7.S Swiss Mode). The coverslip was inverted and placed directly onto a 5 µl drop of Mowiol that had been added onto a glass microscope slide. (See Mowiol preparation in General Methods in **Section 2.1.8**). The slides were protected from light and left to dry. After the mounted coverslip edges were sealed with nail varnish, the neutrophils were observed using an upright 710-Z2 Zeiss Confocal

microscope utilizing the 405 nm, 561 nm, and 633 nm lasers for the DAPI, red, and far red channels, respectively. Dr Ann Wheeler assisted in the initial set up with the confocal microscope. Images were captured using a high powered, X40 oil objective with a 1.30 numerical aperture (na) (DIC M27, EC Plan-Neofluar). Zen 2012 software Version 8 was then used to analyse the images.

5.2.3.2. Quantification of Sytox Positive Neutrophils Preparing Neutrophils in Culture

Parallel to the experiments described above, 400 μ l of 100,000 cells per well was added to separate 24-well plates for the quantification of sytox[®] green extracellular, decondensed DNA using an fluorescent Timelapse microscope. Just as before, several different treatments were prepared. Neutrophils were 1) unstimulated (non-treated), or had the addition of 2) 100 nM of PMA-stimulated neutrophils as a positive control 3) 5 μ M of NE inhibitor, 4) 100 nM PMA and 5 μ M NEi together as an additional control, and lastly 5) 0.01 U/ μ l DNase I as a negative control to dissolve extracellular DNA. Duplicate wells were prepared for all reactions, A mixture of sytox[®] green and Hoechst 33342 dyes (Life Technologies, UK) as prepared by adding 2 μ l of neat (5 mM) sytox green (and 15 μ l of neat (10 mg/ml) of the Hoechst 33342 into 14,983 μ l of culture media. 450 μ l of the mixture was added to the culture well's 450 μ l volume giving a final dilution of 1:15,000 of sytox[®] green (equivalent to 0.33 μ M) and 1:2000 for the Hoechst 33342 (or 5 μ g/ml).

5.2.3.2.1. The Quantification of Sytox Positive Neutrophils in Live Cultures

In-well images were taken of the neutrophils immediately after the addition of the sytox[®] green and Hoechst 33342 mixed using an inverted, epifluorescent Timelapse microscope paired with MetaMorph software (Version 7.7.3.0). The cells were visualised using the X20 air objective. Simultaneous images were captured in each in the DAPI and green fluorescent protein (GFP) channel for at least 3 separate fields of vision.. The images were further analysed using FIJI/ImageJ free software (Schindelin et al., 2012). The number of neutrophils staining positive for sytox[®] green (extracellular DNA) and positive for Hoechst 33342 (live cell nuclear stain) were counted for each individual image. The number of sytox positive cells $\geq 100 \mu\text{m}^2$ area were tallied and divided by the total number of neutrophils (Papayannopoulos et al., 2010). Each image was processed individually and the average percentage (%) of sytox positive (sytox⁺) cells were determined for at least three images of the same treatment and time point for each patient or HC.

In order to count the number of sytox⁺ neutrophils and the total cell number, a threshold was set to automatic pick up grey intensities emanating from the cells so that operator bias was reduced to a minimum (**Fig 5.2.3.2.1.1**). However, any features in the images such as floating cells or light reflection were excluded by manual adjustment to reduce the threshold slightly in order to include cells and exclude imaging anomalies.

SEE FIGURE 5.2.3.2.1.1.

Fig 5.2.3.2.1.1. Simultaneous Images in GFP and DAPI Channels. Sytox[®] green attaches exclusively to extracellular DNA while Hoechst 33342 is able to penetrate the nuclear membrane and stain intracellular, intact DNA. Using FIJI/ImageJ software, minimum thresholds would be automatically set to recognise positively stained cells in each channel. Triplicate images were tallied to determine the mean percentage of sytox⁺ cells for each culture treatment. X20 magnification using air objective on Timelapse microscope. Scale bar 25 μ m.

5.2.4. Patients Included in the Study

BD patients who were experiencing relapsed BD symptoms (n=7) and those in remission (n=3) were included in the study. Four HCs were also included. **Table 5.2.4.1.** provides patient participants' details in the NETs Experiments.

Groups	Subj	Age	Sex	Oral	Gen	Eyes	Jnt	Fol	EN	Vas	CNS	Neutro	Meds
HC	1	33	F	-	-	-	-	-	-	-	-	n/a	None
	2	32	M	-	-	-	-	-	-	-	-	n/a	None
	3	33	F	-	-	-	-	-	-	-	-	n/a	None
	4*	43	F	-	-	-	-	-	-	-	-	n/a	None
BD Non-Relapsed	5	47	F	-	-	-	+	-	-	-	-	5.8x10 ⁹ /L	Aza, NSAID
	6	46	F	+			+	-	-	-	-	5.4x10 ⁹ /L	Ome
	7	43	F	-	-	-	-	-	-	+	-	n/a	MMF, Thal, Ome
	8	60	F	-	-	-	+	-	-	-	-	n/a	Pred
	9	32	F	+	-	-	+	-	-	-	-	2.6x10 ⁹ /L	Doxy, Ome
	10	70	F	-	-	-	+	-	+	-	-	2.6x10 ⁹ /L	NSAID
	11	73	F	-	-	-	+	-	-	-	+	2.5x10 ⁹ /L	None
BD Relapsed	12	32	F	-	-	-	+	+	-	-	+	9.1x10 ⁹ /L	C.std inhaler
	13	33	M	+	-	-	+	-	-	-	-	4.2x10 ⁹ /L	Aza, Colc
	14	41	M	-	-	-	+	+	-	-	+	6.5x10 ⁹ /L	Aza, Colc

Table 5.2.4.1. Patient Demographics for PFA-Fixed NETs Analysis. Symptom abbreviations: **Gen**, genital ulcer. **Jnt**, joints. **EN**, erythema nodosum. **Fol**, Folliculitis. **Vas**, vascular. **CNS**, central nervous system. Medication abbreviations: **Aza**, Azathioprine. **Colc**, Colchicine. **C.std inhaler**, Corticosteroids. **Doxy**, Doxycycline. **MMF**, Mycophenolate mofetil. **NSAID**, non-steroidal anti-inflammatory drug. **Ome**, Omeprazole. **Pred**, Prednisone. **Thal**, Thalidomide. Other: **Neutro**, neutrophil count from clinic (normal range 2-7.5x10⁹/L (Waugh et al., 2006)). Some blood counts were not completed (**n/a**). **M**, male. **F**, female. **+**, active symptoms. **-**, no symptoms. *HC4 did not have enough neutrophils for all staining protocols/culture conditions however sytox green and MPO/NE staining analysis completed.

5.3. Results

5.3.1. Investigating Neutrophil Extracellular Traps in Behçet's Disease: Sytox[®] Green Extracellular DNA Analysis

5.3.1.1. Immediate versus Next Day Imaging

Once sytox[®] green was added, images were immediately taken of the neutrophils while they were still in the culture wells. In order to ease time pressure and improve the practicality of having more time to take images of the cells in culture, images were repeated the next day within 17-24 hours. It was found that there was a significant increase ($p < 0.034$) of the mean percentage of sytox⁺ neutrophils in the PMA-stimulated cultures when the images were taken up to 24 hrs after sytox[®] green was added (**Fig. 5.3.1.1.1**). This presented a problem for the accurate interpretation of sytox⁺ neutrophils. Based on the evidence that PMA-stimulated neutrophils showed an increase in sytox⁺ cells, it was decided that all images should be taken immediately after set time points as opposed to delaying until the next day.

SEE FIGURE 5.3.1.1.1.

Fig. 5.3.1.1.1. A. and B. Representative Results of Increased Sytox⁺ Neutrophils During Next Day Imaging of the Same 1 hr PMA-Stimulated HC Neutrophil Culture. **A.** Top images are of the culture 1 hr after cells were stimulated with 100 nM PMA. Images were taken within 1 hr of adding sytox[®] green and Hoechst DNA stains to the culture media with live neutrophils. Bottom images were taken from the same HC PMA-stimulated culture within 24 hrs. **B. The Mean Difference Between Sytox⁺ HC Neutrophils when Imaging the Same Culture at Different Times.** Error bars represent \pm SEM of the mean percentage of sytox positive cells ($\geq 100 \mu\text{m}^2$) from multiple images of biological replicates. Mann Whitney U, 2-tailed, non-parametric test, * $p < 0.05$.

The unstimulated neutrophils seemed to absorb more Hoechst nuclear stain during the long incubation overnight as the fluorophore was more intense the next day (**Fig 5.3.1.1.2, A**) but this did not alter the ability to accurately detect and count the cells. Because unstimulated HC neutrophils showed similar sytox green results with a difference of only 0.14% between immediate and next day imaging (**Fig 5.3.1.1.2, B**) the data for this group and culture condition was included in the subsequent analysis. However, all other HC and BD patient neutrophil culture treatments were assessed immediately.

SEE FIGURE 5.3.1.1.2

Fig. 5.3.1.1.2, A. and B. An Example of Increased Sytox⁺ Neutrophils During Next Day Imaging of the Same Unstimulated HC Neutrophil Culture. A. Top images were taken within 1 hr of adding Sytox green and Hoechst stains to the culture media with live neutrophils. Bottom images were taken from the same HC culture within 24 hours. **B. The Mean Difference Between Sytox⁺ HC Neutrophils when Imaging the Same Culture at Different Times.** Error bars represent \pm SEM of mean percentage of sytox positive cells ($\geq 100 \mu\text{m}^2$) from multiple images of biological replicates.

5.3.1.2. Sytox® Green Analysis in Different Culture Conditions

5.3.1.2.1. Positive Control Experiments: Inducing Neutrophils Extracellular Traps using PMA Stimulant

The first experiment was to determine how many neutrophils were stimulated using PMA to produce NETs in HC and BD patients' neutrophils. The neutrophils from each patient were subjected to a positive control experiment in which 100 nM of PMA was added to the cultures in duplicate after an hour of being settled in the media. All patients' cultures were able to induce NETs, as determined by the extracellular DNA staining with sytox[®] green. NET production increased by 13.4% (median value) in BD relapsed patients' neutrophils when subjected to PMA stimulation (**Fig 5.3.1.2.1.1**). This was nearly triple the difference seen in non-relapsed patients whose incidence of NETosis rose only by 4.6%. The smallest difference was observed in HC neutrophils in which unstimulated cells showed 0.36% of the cells undergoing NETosis rising to 2.5% with PMA stimulation.

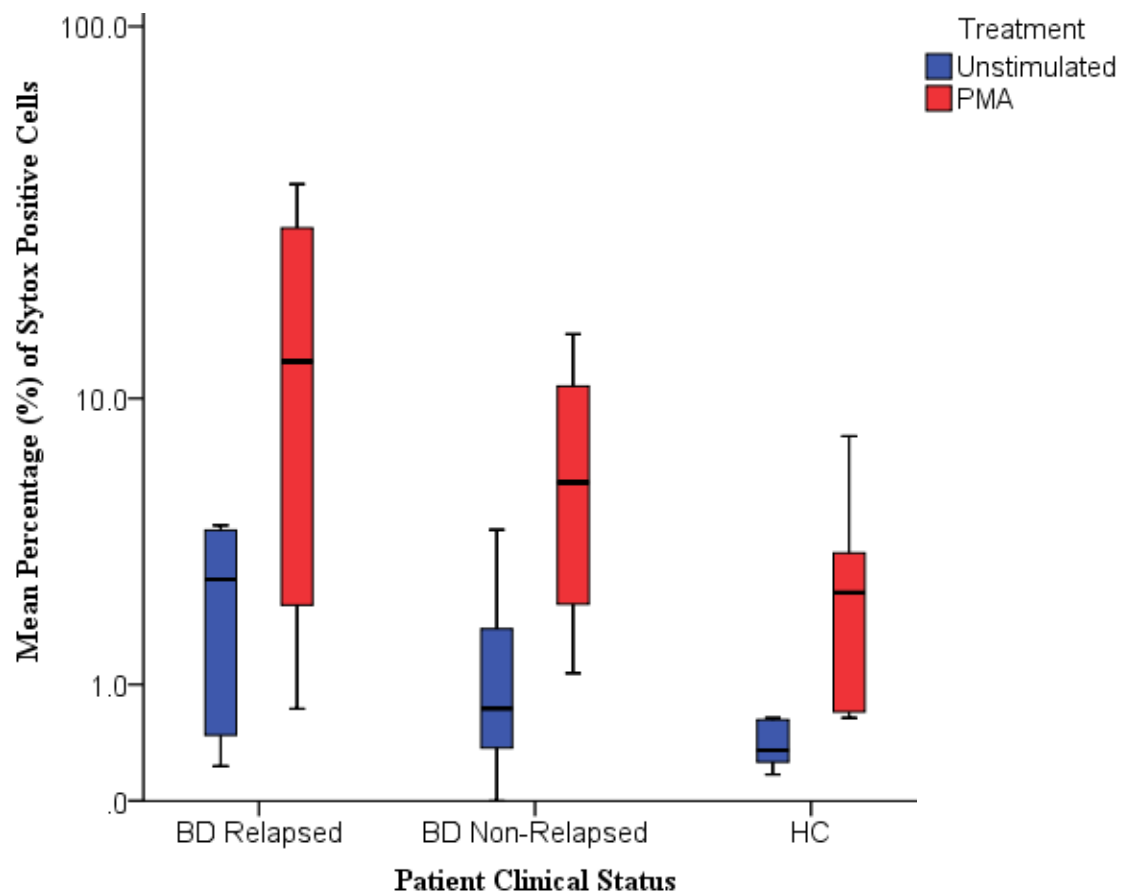


Fig 5.3.1.2.1.1. Inducing NETosis *In Vitro* using PMA: A Positive Control Experiment. Comparing the median group value of the mean percentage (%) of sytox⁺ neutrophils in unstimulated and PMA-stimulated cultures. The data in this figure stems from images taken immediately after adding sytox[®] green to the cultures. Both time points are included.

When comparing the post-PMA stimulation time points, it was determined that the production of NETs increased by 27.2% (median group value) in BD Relapsed patients from the first to second time point. The non-relapsed BD patients only increased 9.21% while the HC showed the lowest increase at 2.68% between time points (**Fig 5.3.1.2.1.2**).

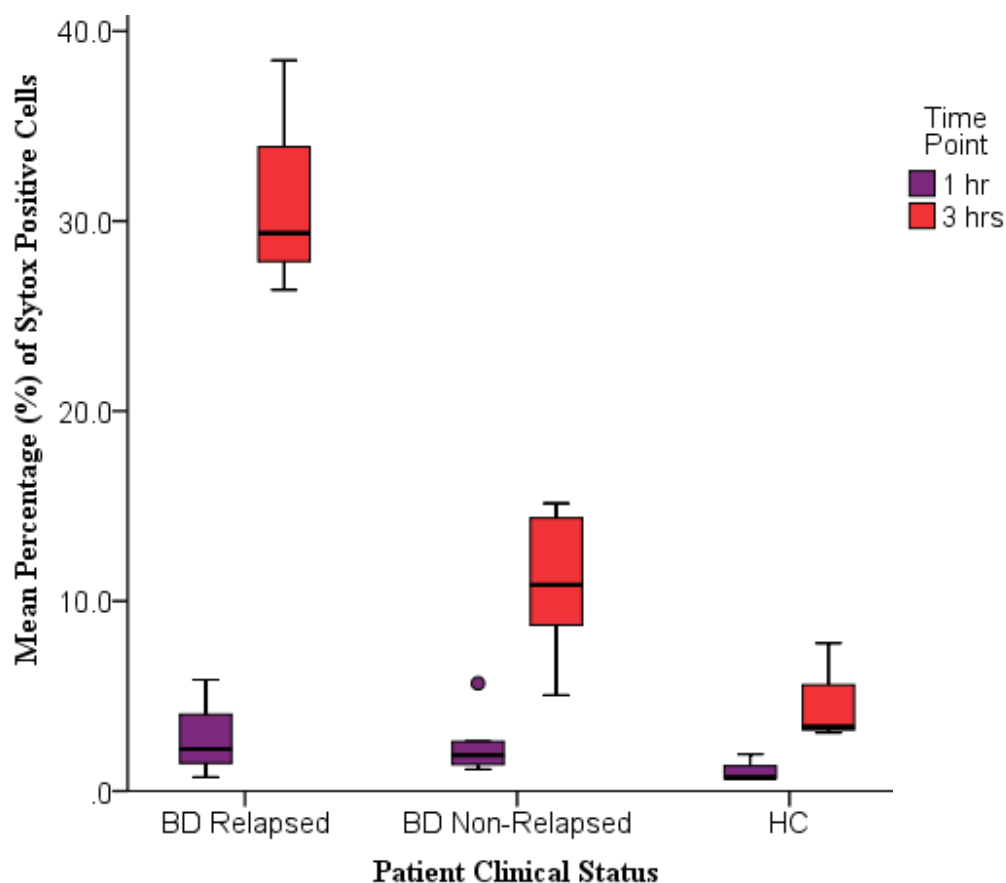


Fig 5.3.1.2.1.2. The Difference in Time Points when Inducing NETosis *In Vitro* using PMA. Comparing the median group value of the mean percentage of sytox⁺ neutrophils PMA-stimulated cultures at the first (1 hr post-stimulation) and second time point (3 hrs post-stimulation). The data in this figure stems from images taken immediately after adding sytox[®] green to the cultures.

5.3.1.2.2. Comparing Unstimulated Sytox+ Neutrophils from Different Patient Groups in Vitro

Unstimulated cultures refer to neutrophils which were not exposed to any known artificial chemical or pathogenic stimulant in the culture wells. In these unstimulated cultures, there was a significant increase in the detection of extracellular DNA due to the formation of NETs in the BD patient group, regardless of whether they were experiencing disease relapse or not, compared to HC, $p=0.02$ (**Fig 5.3.1.2.2.1**). With the exception of one measurement, all HC neutrophil cultures had $< 1\%$ sytox⁺ (Mean, SEM; $0.5, \pm 0.12$). Meanwhile, collectively, BD patients had an average of 1.5% (± 0.32) which was triple the HC average.

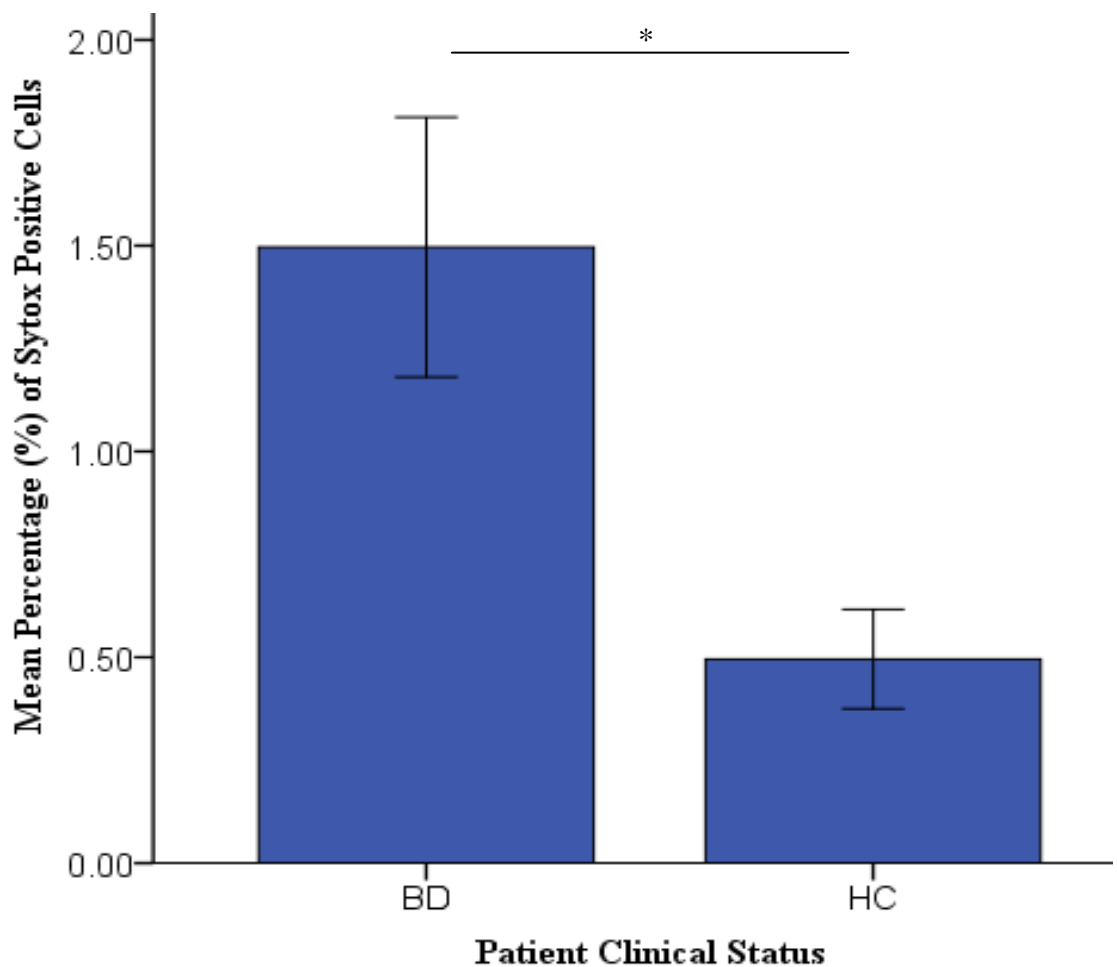


Fig 5.3.1.2.2.1. The Mean Percentage of Sytox Positive Neutrophils in HC and BD patients. Data includes two time point measurements (2 hrs and 4 hrs in culture) from unstimulated cultures and HC images that were taken immediately. Subjects tested: HC $n=4$, BD $n=10$. Total number of mean % sytox⁺ cell measurements: HC=14, BD=20. Mann Whitney U non-parametric analysis; * indicates $p \leq 0.05$. Error bars represent ± 1 SE.

BD patients who were considered to have a clinically defined relapse of symptoms showed significantly higher sytox⁺ cells than HC (p=0.032) (**Fig 5.3.1.2.2.2.**). BD relapsed patients also showed a higher percentage of sytox⁺ cells than non-relapsed patients but was not significant (p=0.149). Although the non-relapsed BD group had a higher mean percentage of sytox⁺ cells than HC, the difference was also not significantly (p=0.069).

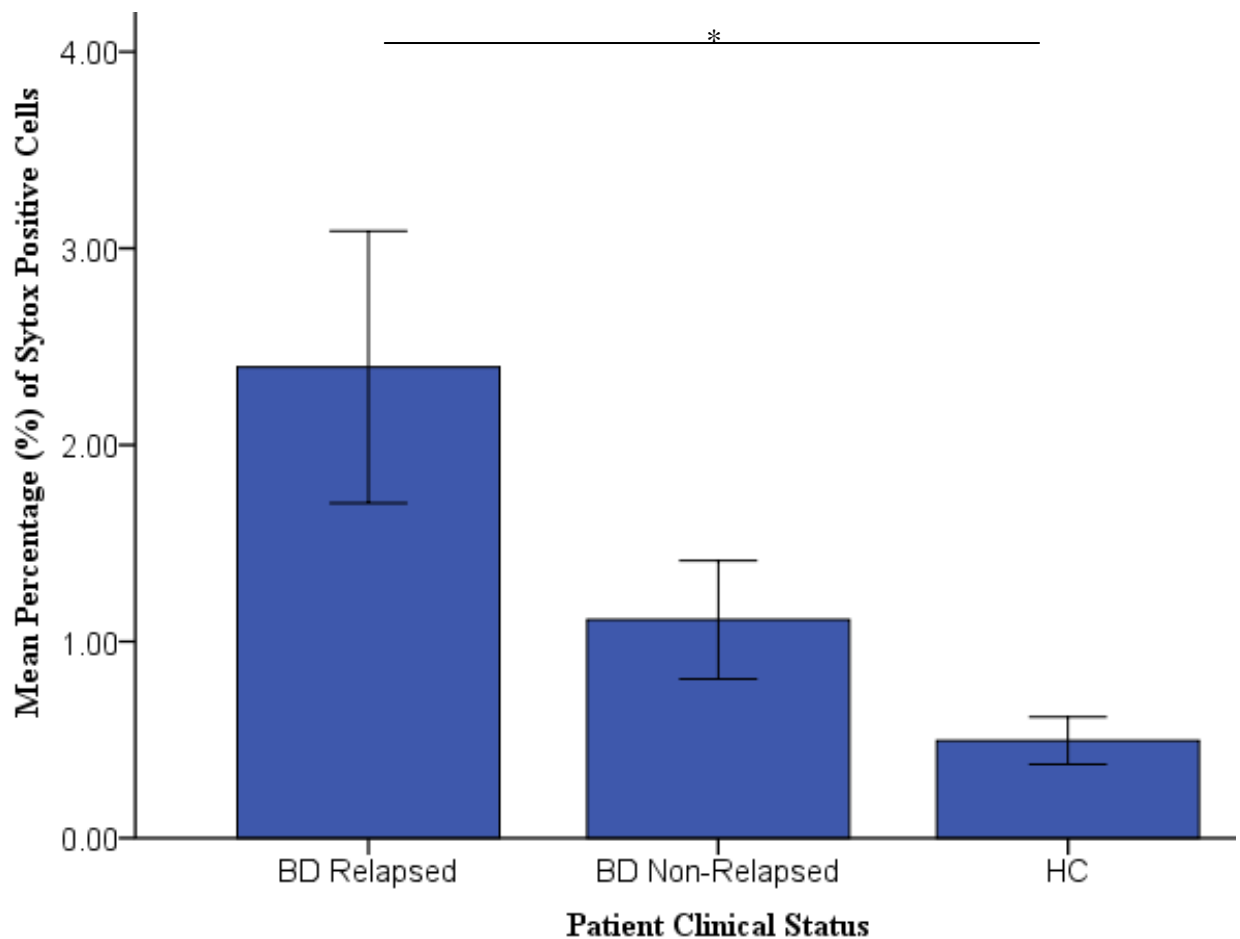


Fig 5.3.1.2.2.2. The Mean Percentage of Sytox Positive Neutrophils in Clinically Relapsed and Non-Relapsed BD Patients Compared to HC. Data combines two time point measurements (2 hrs and 4 hrs in culture) from unstimulated cultures. Number of subjects tested: BD Relapsed n=3, BD Non-Relapsed n=7, HC n=4. Number of mean % sytox⁺ cell measurements in the data analysis: BD Relapsed=6, BD Non-Relapsed=14, HC=14. Mann Whitney U non-parametric analysis; * indicates p≤0.05. Error bars represent ± 1 SE.

All BD patients on azathioprine (Aza) medication for the treatment of their symptoms revealed a higher group median of the mean percentage of sytox⁺ cells than patients not taking the drug, however the differences were not significant (p=0.083). BD relapsed patients taking Aza had a group median of 3.44% (of the mean percentage of sytox⁺ cells) while those with the same status not taking Aza were more than half the value at 1.56%. (p=0.36) (**Fig 5.3.1.2.2.3**). Similarly, comparing the median values of the non-relapsed patient on Aza (1.77%) to non-relapsed patients not taking the medication (0.61%) was again reduced by more than half the median value (p=0.20). Only two out of 10 BD patients were on colchicine and therefore were not assessed.

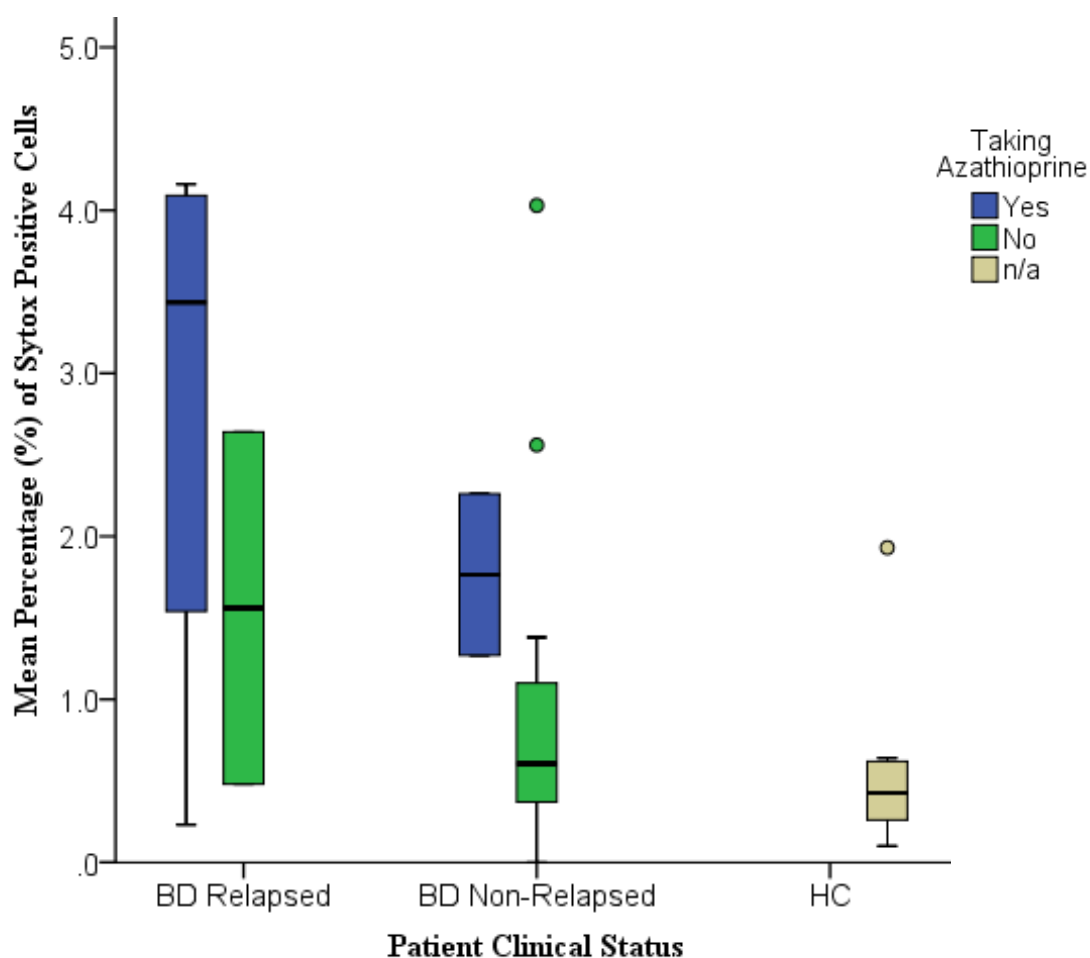


Fig 5.3.1.2.2.3. The Mean Percentage of Sytox⁺ Neutrophils: Comparing Patients On Azathioprine Medication. The box plot shows the median group values of the mean percentage of sytox⁺ neutrophils of patients who were on Azathioprine (Aza) at the time blood was donated. The data includes two time point measurements from unstimulated cultures (2 hrs and 4 hrs). The box itself represents 50% of the values in the group and the extended bars indicate maximum and minimum values. Circles indicate outliers. Number of subjects (n) and mean % sytox⁺ measurements represented in the data: BD Relapsed on Aza, n=2, sytox⁺=4; BD Relapsed not on Aza n=1, sytox⁺=2; BD Non-Relapsed on Aza n=1, sytox⁺=2; BD Non-Relapsed not on Aza n=6, sytox⁺=12. HC not taking any medication, n=4, sytox⁺=14. Mann Whitney U, non-parametric analysis.

Data from the first and second time points represent duplicate neutrophil cultures that had been incubated at 37°C with 5% CO₂ for a total of 2 and 4 hrs, respectively. BD Relapsed patients showed a high variation of the mean % of sytox⁺ neutrophils after the first 2 hrs in culture (ranging from 0.23 to 4.16%) (**Fig 5.3.1.2.2.4**). This produced a low median group value of 0.48%. However, after 4 hrs in culture, the median group value for the relapsed patients increased to 2.85%. Due to the 2 hr time point data variability and low number of BD relapsed patients, this resulted in no significant difference between 2 and 4 hrs (p=0.51). Similar medians were determined for both time points in the BD Non-Relapsed patients group. After 2 hrs in culture, the group's median (of the mean % of sytox⁺ neutrophils) was 0.65%. After 4 hrs in culture, this occurrence was sustained resulting in a similar median value of 0.61%, however it did show greater variability. Lastly, after 2 hrs in culture, neutrophils from the HC group resulted in a median value of 0.41% (of the mean % of sytox⁺ neutrophils). The median increased only slightly to 0.44% after 4 hrs. The only significant difference found between median values were the 4 hr cultures from BD Relapsed and HC groups (p=0.016) (**Fig 5.3.1.2.2.4**). In general the data showed that after a longer incubation in unstimulated culture, there was an increase in the mean % of sytox⁺ neutrophils in BD patient groups.

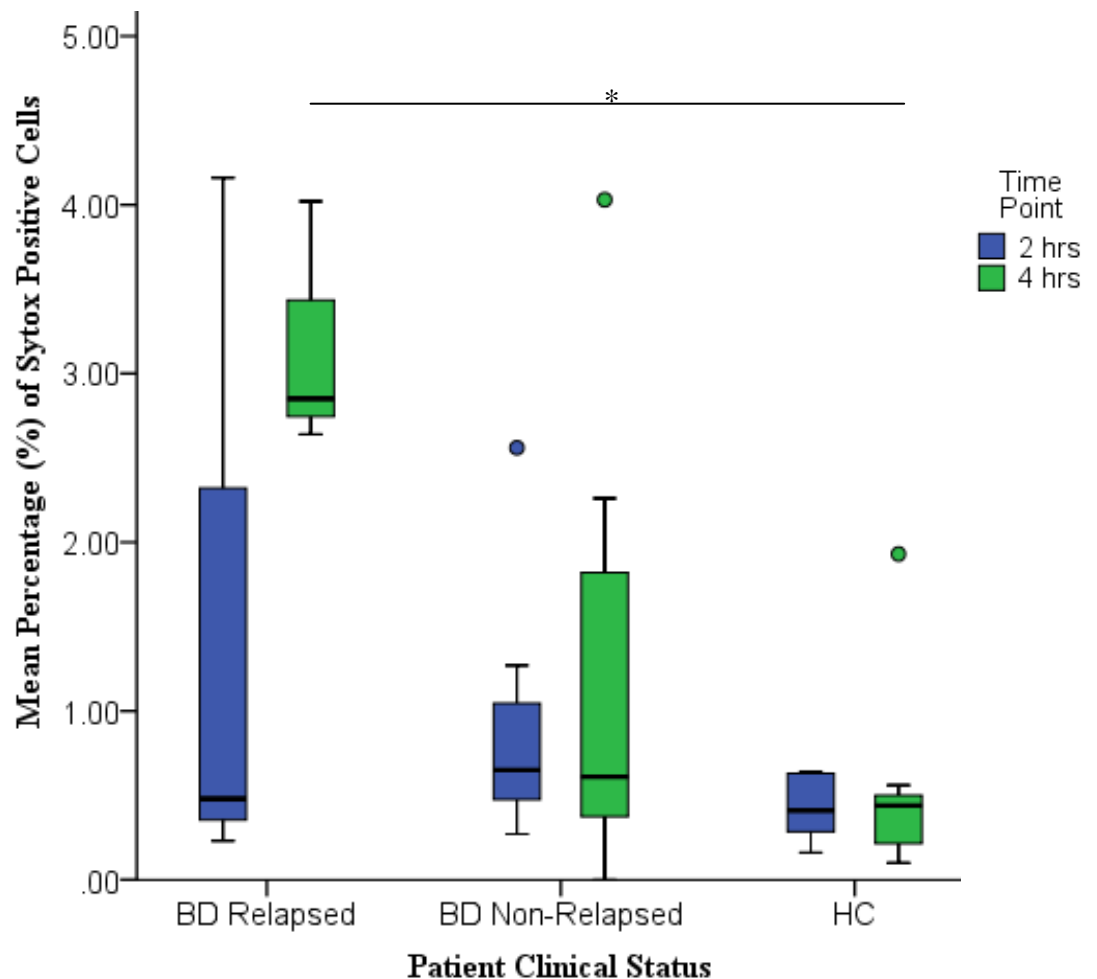


Fig 5.3.1.2.2.4. Comparing the Mean Percentage of Sytox⁺ Neutrophils in Unstimulated Cultures at Two Different Time Points. The median group values of the mean percentage of sytox⁺ neutrophils were determined for both time points (2 hrs and 4 hrs after initial plating) when the cultures were stained with sytox[®] green and Hoechst and immediately imaged. The bar in the centre of each box indicates the median group value. The box itself represents 50% of the values in the group and the extended bars indicate maximum and minimum values. Circles indicate outliers. Number of subjects included: BD Relapsed n=3, BD Non-Relapsed n=7, HC n=4. Number of mean % sytox⁺ cell measurements for both time points: BD Relapsed=6, BD Non-Relapsed=14, HC=14. Mann Whitney U non-parametric analysis; * indicates $p \leq 0.05$.

5.3.1.2.3. The Effect of NEi on Neutrophils

When comparing PMA-stimulated cultures with and without NEi, after 1 hr there were no significant changes between any of the patient group medians (of the mean % of sytox⁺ neutrophils). However, cultures with NEi that were exposed to 3 hrs of PMA-stimulation showed significant decreases compared to PMA-only cultures in two patient groups: BD Relapsed ($p \leq 0.05$) and Non-Relapsed ($p < 0.02$) (**Fig 5.3.1.2.3.1**). This indicated that NEi had the desired affect to inhibit the occurrence of NETosing neutrophils thus lowering the number of sytox⁺ neutrophils after a longer time *in vitro*. BD Relapsed patients' showed the largest decrease between PMA-only and PMA with NEi cultures at 23.3% followed by 8.5% for the BD Non-Relapsed group. The group median in PMA-stimulated HC cultures also decreased by 2.1% when NEi was included but this was not a significant reduction. (Note that the total time in culture was 2 hrs with 1 hr of PMA-stimulation and 4 hrs with 3 hrs of PMA).

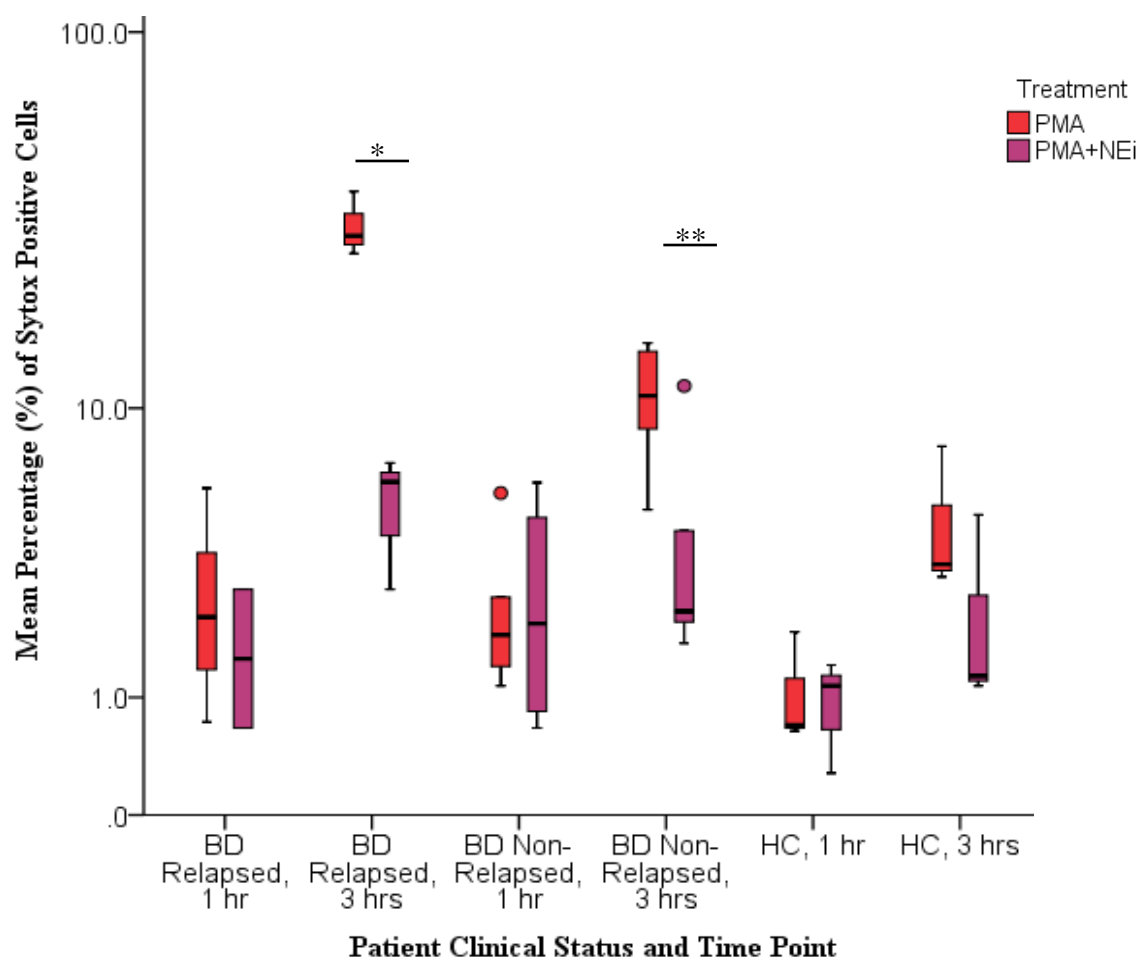


Fig 5.3.1.2.3.1. Comparing the Mean Percentage of Sytox⁺ Neutrophils in 1 hr and 3 hrs PMA-Stimulated Cultures with and without Neutrophil Elastase Inhibitor (NEi) in Different Patient Groups. The median group values of the mean percentage (%) of sytox⁺ neutrophils were determined for PMA-stimulated cultures with and without 5 μ M of NEi. The 1 hr and 3 hrs indicate the time the cultures were stimulated with PMA. The bar in the centre of each box indicates the median group value. The box itself represents 50% of the values in the group and the extended bars indicate maximum and minimum values. Circles indicate outliers. Number of subjects included: BD Relapsed n=3, BD Non-Relapsed n=6, HC n=3. Data are expressed on a log scale. Mann Whitney U, 2-tailed, non-parametric test, *p \leq 0.05, **p \leq 0.02.

Both HC and BD non-relapsed patient neutrophil cultures with NEi had slightly higher group median (of the mean % of sytox⁺ cells) than the unstimulated cultures at the same time point. The only group that showed a reduction in sytox⁺ neutrophils with the addition of NEi was the relapsed BD patients after 4 hrs in culture. No statistical differences were found when comparing group medians (**Fig 5.3.1.2.3.2**).

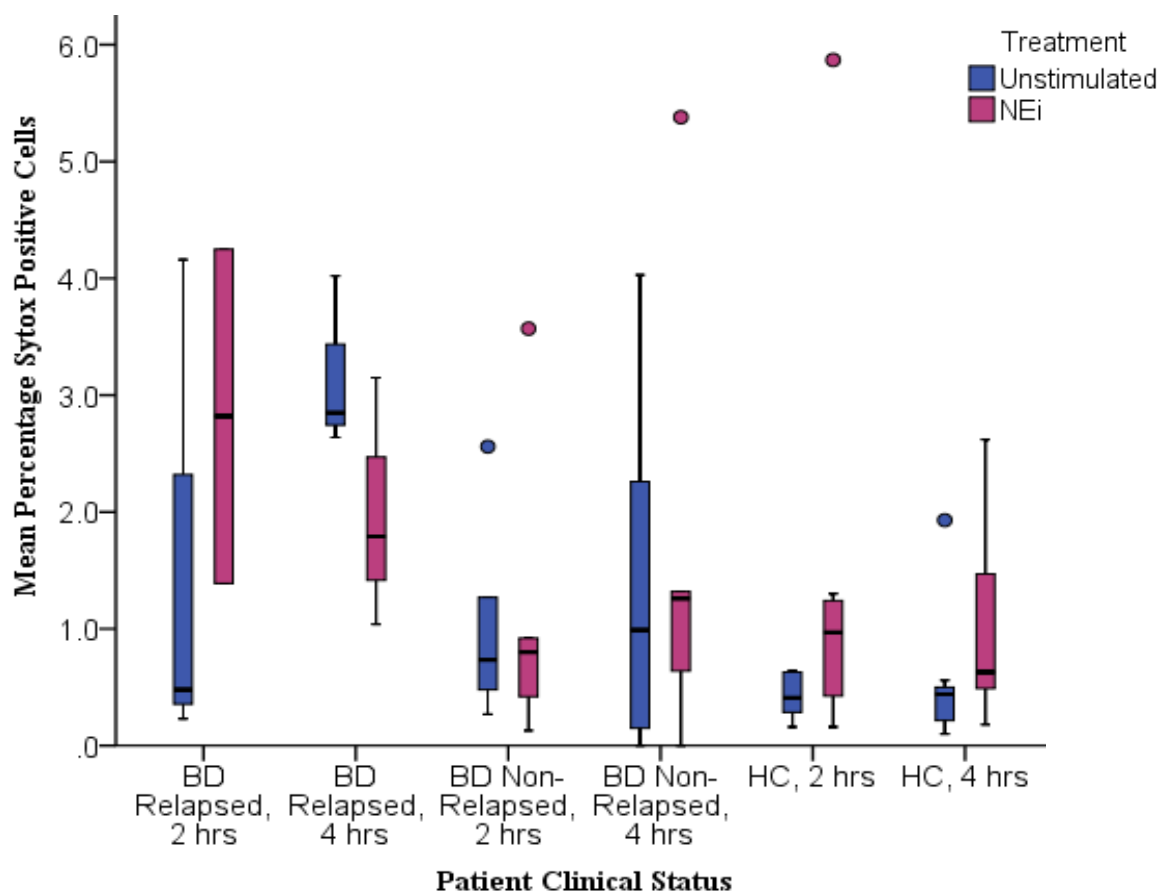


Fig 5.3.1.2.3.2. Comparing the Mean Percentage of Sytox⁺ Neutrophils in Unstimulated and Neutrophil Elastase Inhibitor (NEi) Treated Cultures in Different Patient Groups. The median group values of the mean percentage of sytox⁺ neutrophils were determined for both time points of unstimulated and NEi cultures. The bar in the centre of each box indicates the median group value. The box itself represents 50% of the values in the group and the extended bars indicate maximum and minimum values. Circles indicate outliers. Number of subjects included: BD Relapsed n=3, BD Non-Relapsed n=6, HC n=3. Mann Whitney U, non-parametric analysis.

5.3.2. NETosis with and without PMA-Stimulation: Observation of Neutrophil Elastase *In Vitro* Using Immunocytochemistry

Neutrophil elastase (NE) has been recognised as a key enzyme involved in the NETosis cell death pathway. Characteristically, NE is seen to translocate from its azurophil granules located throughout the cytoplasm and enter the nucleus due to the loss of nuclear membrane integrity. NE then mixes with the cell's nuclear material unravelling the coiled nucleosome's DNA and histone complex that configure compact chromatin. In order to demonstrate the phases of NETosis, **Fig 5.3.2.1** is a collection of images from our study showing PMA-stimulated neutrophils undergoing NETosis *in vitro* similar to how it has been reported in previous publications.

For this study, the images in **Fig 5.3.2.1** were categorised as three separate stages of the NETosis process: early, intermediate, and advanced. But to begin with, in **Fig 5.3.2.1, panel 1** shows the classic multi-lobed, hypersegmented, round-bodied neutrophil. There is a clear separation of the NE in the cytoplasm (contained in its azurophil granules) and the DNA in the nuclear envelope. NE defines the early NETosis process by leaving its granules and gaining entry to the nucleus where it breaks down DNA-histone complex causing enlargement of the nuclear area. Meanwhile, the granules seem to become more concentrated as the staining is more pronounced throughout the cytoplasm (**Fig 5.3.2.1, panel 2**). A few prominently stained NE granules remained in the cytoplasm (**Fig 5.3.2.1, panel, 3**). As the cell entered the intermediate stage of NETosis, the hypersegmented structure of the neutrophil's nucleus completely dissipates (**Fig 5.3.2.1, panel 4**). Vacuoles appear within the remaining nuclear material and the cytoplasm, however, the cell's plasma membrane is still intact (**Fig 5.3.2.1, panel 5**). Finally, in the advanced stage, the cell's membrane loses its integrity and the internal contents disperse into the external space (**Fig 5.3.2.1, panel 6**).

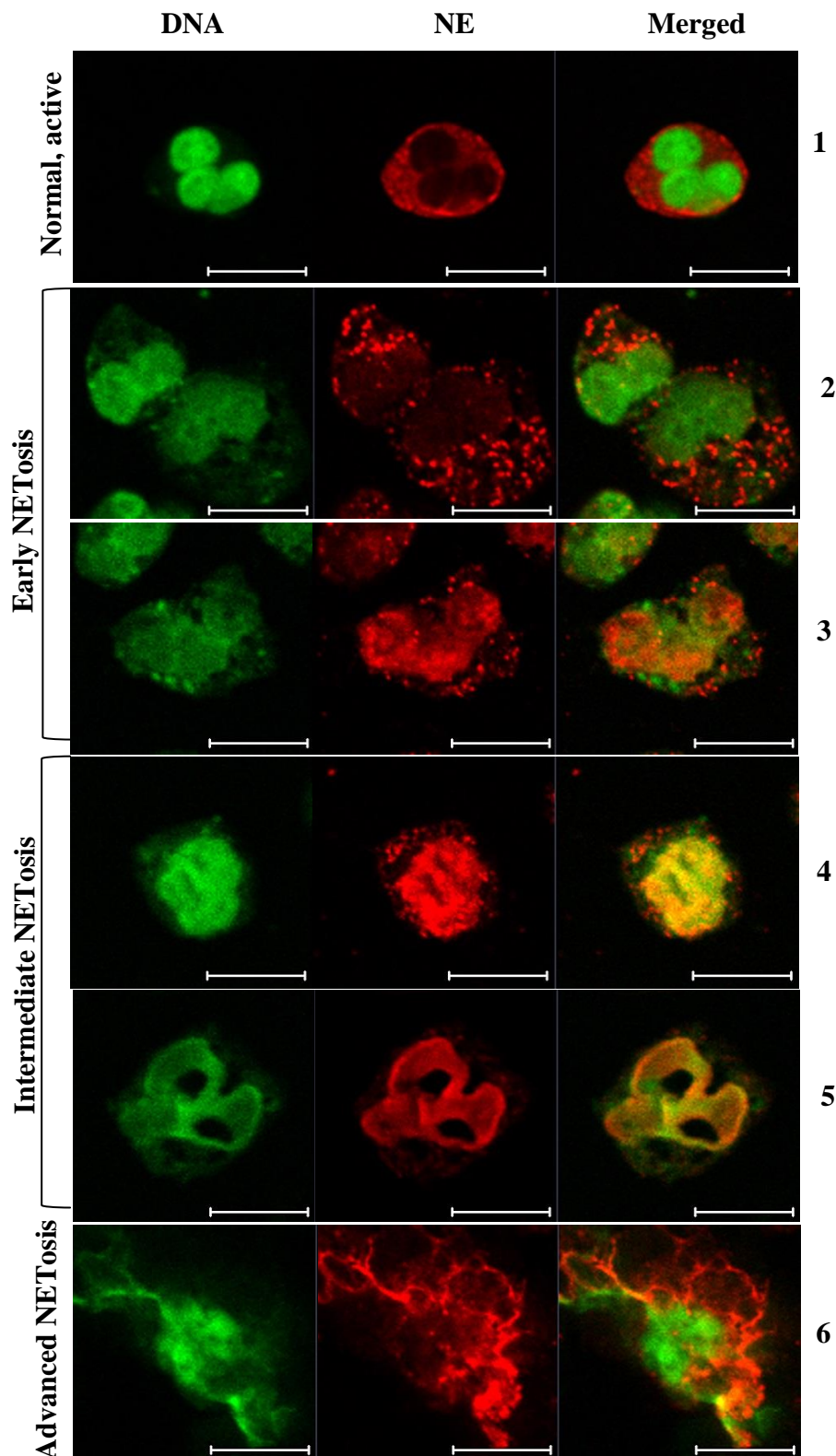


Fig 5.3.2.1. Positive Control: Neutrophils Undergoing NETosis in a PMA-Stimulated Culture. Panels show the typical neutrophils morphologies undergoing NETosing following PMA stimulation. Panel 1 shows a normal, hypersegmented neutrophil. Panels 2 and 3 show early NETosis in which granules became more pronounced. This stage is defined by the initial translocation of NE (red) into the nucleus (DNA, green). This co-localisation creates a yellow hue. DNA decondensation takes place. Panels 4 and 5 show neutrophils undergoing the intermediate stage in which the nucleus continues to swell and vacuoles develop. Panel 6 shows the advanced stage of NETosis in which cells have erupted and cast out NETs. Confocal 710, X40 oil magnification. Bar indicated 10 µm.

Blood neutrophils from HC (n=3 for SLPI staining, n=4 for MPO staining), non-relapsed BD (n=7), and relapsed BD patients (n=3) were investigated *in vitro* to determine whether they would naturally undergo NETosis without PMA-stimulation. Observations of the different stages of NETosis displayed in **Fig 5.3.2.2** were assessed by confocal microscopy after immunocytochemical staining. The naturally occurring NETosis had some distinguishing features that were different from the PMA-stimulated process. In early NETosis, NE was seen to mix with the cell's DNA while maintaining some of the hypersegmented morphology, however the azurophil granules were not as prominent in the cytoplasm as they were with PMA (**Fig 5.3.2.2, panel 1 and 2**). This was evident for both HC and BD patients. In fact, NE was seen to frequently co-localise with the entire nucleus (**panel 2**) leaving no evidence of NE remaining in the cytoplasm. Some of the nuclei were seen to enlarge but not as much as the PMA-induced cells. Total decondensation of the DNA took place during the intermediate stage (**Fig 5.3.2.2, panel 3**). Similarly to PMA, vacuoles could be seen through-out the cell while the plasma membrane remained intact (**Fig 5.3.2.2, panel 4**). Finally, expulsion of the cell contents marked the advanced stage of NETosis (**Fig 5.3.2.2, panel 5 and 6**). The majority of NE and DNA fibres were cast into the immediate surroundings without any predictive formation, however some of the extracellular fibres were distinctively different compared to the PMA-induced NETs (**Fig 5.3.2.2, panel 5**). The NET itself looked highly structured with a thick mesh, fan-like structure. This occurred in two different BD patients' unstimulated cultures.

A histone antibody targeting one of the core proteins, H3, was included in some initial staining. However, the staining was discontinued because H3 and DNA staining were identical (data not shown) and it was concluded that the DAPI DNA stain was sufficient for the detail required in these experiments.

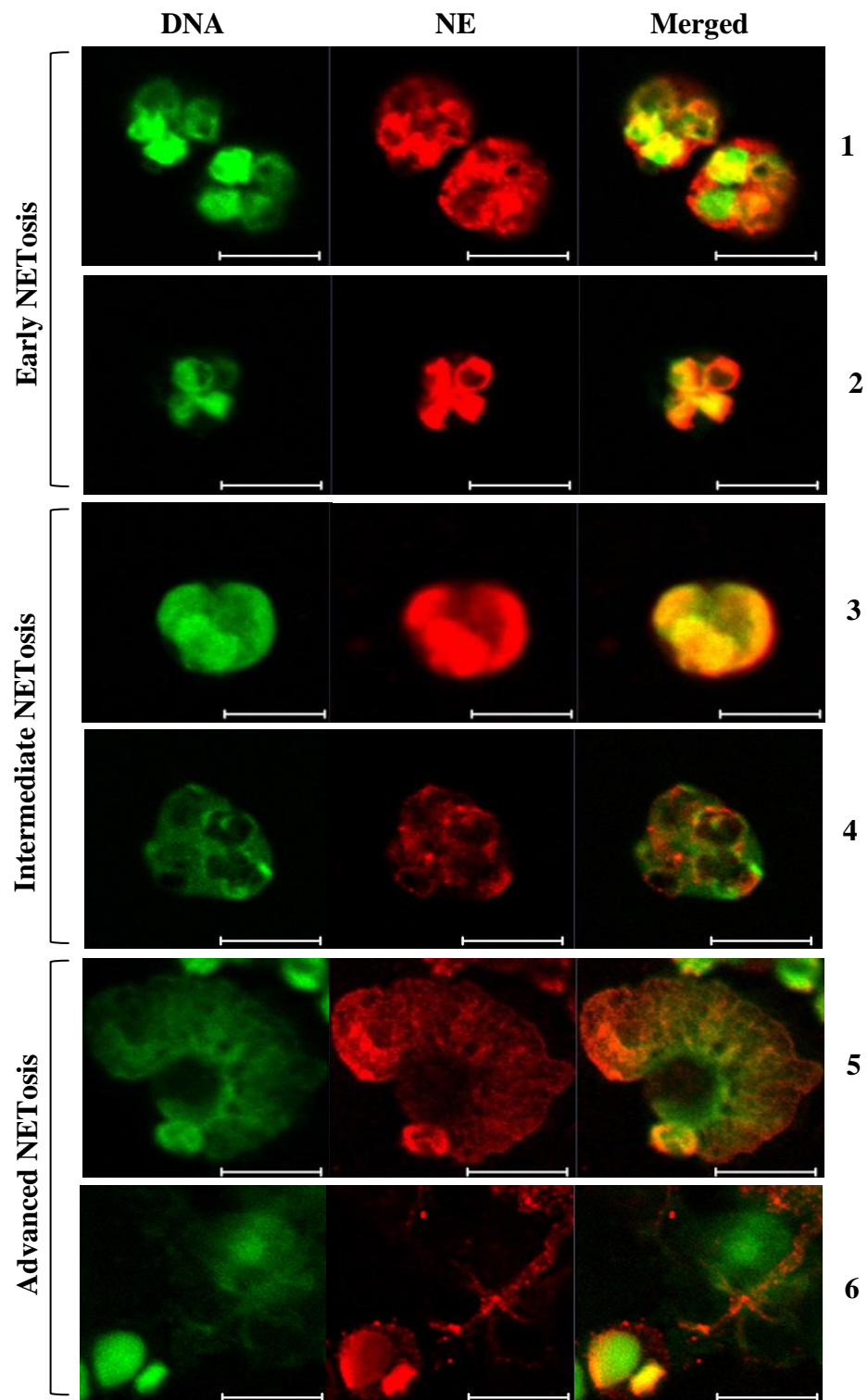


Fig 5.3.2.2. BD Patients' Neutrophils Undergoing NETosis in Unstimulated Culture. The panels show the various morphologies of NETosing neutrophils. The co-localisation of NE (red) and DNA (green) created a yellow hue. Incubations in culture media were up to 4 hours. Images taken on Confocal 710, X40 oil magnification. Bar indicated 10 µm.

5.3.2.1. NE Inhibitors and NETosis

5.3.2.1.1. NE and its Synthetic Inhibitor

After initially observing NE's role during induced and naturally occurring NETosis, it was important to further investigate the same cultures with NE inhibitor (NEi). This was conducted in order to determine whether: 1) NE was an integral element in NETosis and if so, 2) whether HC and BD patients' neutrophils undergoing NETosis could be repressed with NEi. The inhibited NETosis reaction expected due to the introduction of NEi is demonstrated in **Fig 5.3.2.1.1**. In the unstimulated cultures, it was hypothesised that pre-activated isolated blood neutrophils would undergo NETosis, but with the addition of NEi, the process would be inhibited. The same dampening of NETosis cell death was expected in the PMA-stimulated cultures with NEi. Eight out of 14 (57.1 %) individuals showed this anticipated reaction to 5 μ M of NEi in at least one of their time point incubations. These included 2 out of 4 HCs, 2 out of 3 BD Relapsed patients, and 4 out of 7 BD Non-Relapsed patients. All of these cultures were observed almost exclusively during the later time point which was after 4 hrs of normal incubation conditions or 3 hrs of PMA exposure. The only one that showed this hypothesised reaction after 2 hrs of incubation (or 1 hr of PMA-stimulation) was a BD Relapsed patient.

As shown in **Fig 5.3.2.1.1**, NETosing neutrophils in the unstimulated culture (first panel) changed when NEi was added (second panel) - NEi inhibited the NETosis cell death pathway. The neutrophils maintained their lobulated nuclei, the cell did not enlarge, and NE from the granules did not move into the nucleus. Again this is demonstrated in the third and fourth panels which show PMA-induced NETosis and the NEi inhibited reaction, respectively. Although there was some swelling of the neutrophils and the NE seems more granular in the cytoplasm, the NE did not translocate to the nucleus which remained hypersegmented.

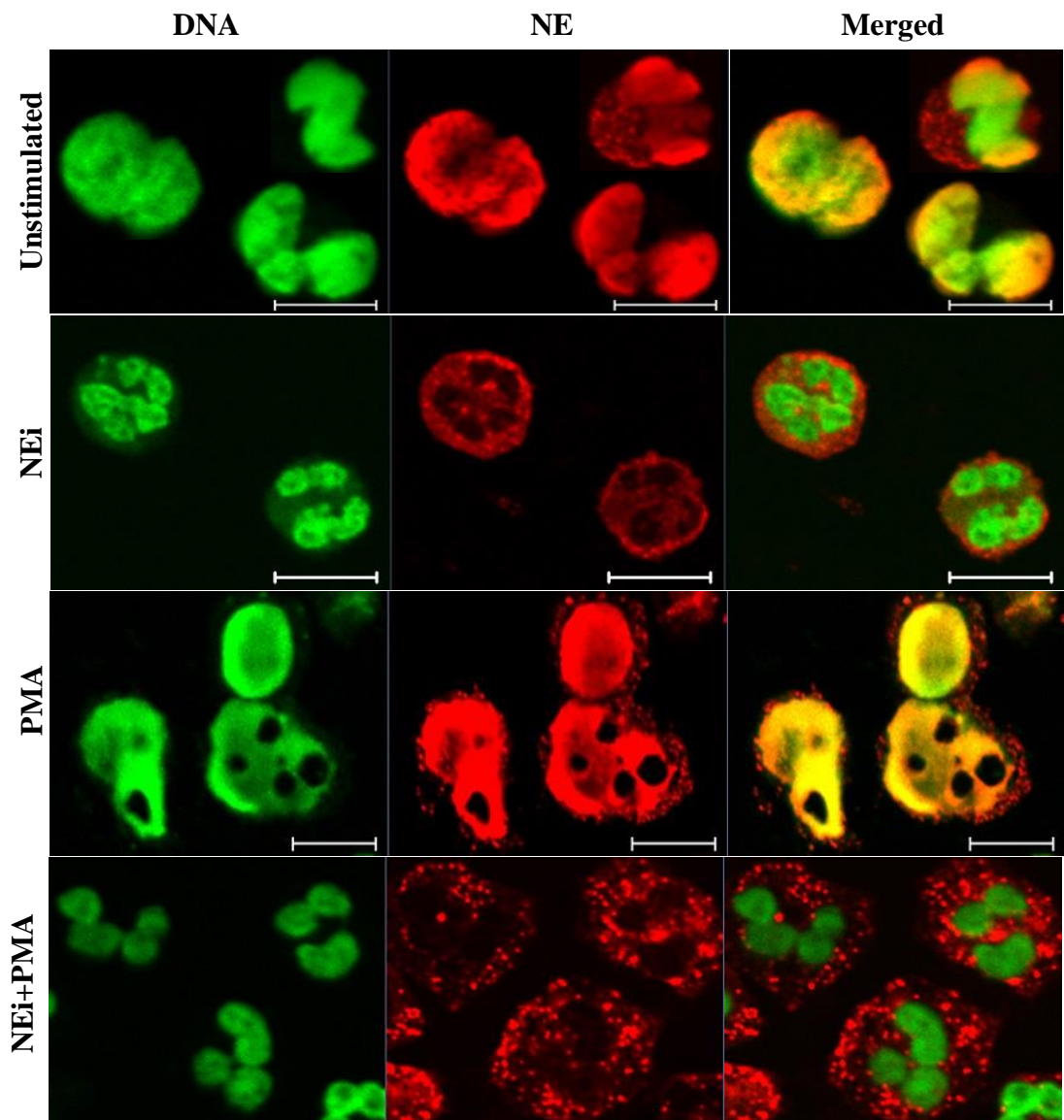


Fig 5.3.2.1.1. The Hypothesized Reaction to NE Inhibitor in Unstimulated and PMA-Stimulated NETosis. The panels show the expected neutrophil reactions in different culture conditions: with or without PMA-stimulation and NEi. The images are neutrophils from a BD patient. The co-localisation of NE (red) and DNA (green) created a yellow hue. Images taken on Confocal 710, X40 oil magnification. Bar indicated 10 µm.

5.3.2.1.2. Endogenous Neutrophil-Derived SLPI During NETosis

In addition to targeting NE to investigate its role in NETosis, the neutrophil's endogenous inhibitor, SLPI, was also observed for any interactions with NE. Each patient group was assessed separately. The neutrophil images in the following panels are a collection of representative cultures from HC or BD patients.

5.3.2.1.2.1. NE and SLPI in Unstimulated Cultures

5.3.2.1.2.1.1. Unstimulated HC Blood Neutrophils *In Vitro*

Very few PFA-fixed, antibody stained neutrophils were seen using confocal microscopy in the cultures from 3 out of 4 HC subjects whose original culture started with $1-4 \times 10^5$ cells. In fact, only 0-2 cells were observed per frame at X40 magnification which is represented by HC1 in **Fig 5.3.2.1.2.1.1, Panel 1**. (The dimensions of each imaged frame on the confocal microscope's X40 objective was $212 \mu\text{m} \times 212 \mu\text{m}$.) Only one HC subject's neutrophils had an average of 7 cells per frame (**Fig 5.3.2.1.2.1.1, Panel 1, HC2**).

Typical, hypersegmented neutrophils were infrequently detected from HCs probably due to a lack of cell priming (i.e. surface ligand activity) and would therefore not adhere as well to the plain glass coverslips. Thus it was presumed that the majority of neutrophils that did not adhere did not encounter a stimulus *in vivo* or *in vitro* (**Smith et al., 1979**) (**Webster et al., 1986**). In **Fig 5.3.2.1.2.1.1, Panel 1** HC neutrophils labelled **a** are some of the few hypersegmented neutrophils seen. In particular, these neutrophils showed small blebbing projections from the main body of the cell which signifies that the cells were either undergoing early apoptosis and/or actively travelling using their pseudopodia-like projections. Apoptosing neutrophils (**Panel 1, label b**) were occasionally observed in the unstimulated cultures. They were identified by condensed nuclei which was no longer hypersegmented and the cell diameter was less than $10 \mu\text{m}$. NE and SLPI could be detected in the cytoplasm separate from DNA.

After 2 hrs of culture, very few HC cells were detected undergoing NETosis as is evident in **Fig 5.3.2.1.2.1.1, Panel 1 (c, d)**. A higher intensity of the NE antibody stain was observed on the border of the still hypersegmented nuclei in early NETosis (arrow, **c**). SLPI coincided with the NE stain and the overlap of all three NE, SLPI, and DNA fluorescent antibodies created a bright pink-white tinge. Advanced NETosis in unstimulated HC neutrophils was rare (**d**). Finally, there were a few non-NETosing $\text{SLPI}^+/\text{NE}^-$ eosinophils (**e**) identified in the cultures as well.

SEE FIGURE 5.3.2.1.2.1.1.

Fig 5.3.2.1.2.1.1. Unstimulated HC Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in Unstimulated Cultures. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs (Panel 1) and 4 hrs (Panel 2) in culture media without NE inhibitor before being fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

After 4 hrs in culture, there were very few cells from HC subjects that remained attached to the coverslips. Some of the cells that did attach were hypersegmented (**Fig 5.3.2.1.2.1.1, Panel 2, HC1**) although these were frequently located closer to the coverslip edge where they were probably agitated. Other cells included a few small neutrophils with condensed DNA that were thought to be apoptosing (**Fig 5.3.2.1.2.1.1, Panel 2, HC2**). Two neutrophils were detected undergoing the early stage of NETosis (**Fig 5.3.2.1.2.1.1, yellow arrows**) in which NE mixed with the DNA with some initial decondensation. In some cells, the NE seemed more concentrated along the periphery of the nucleus. However, even after 4 hrs in culture, the advanced eruption stage of NETosis was only observed once among the unstimulated HC cultures.

SLPI was detected in all cultured neutrophils. In hypersegmented and apoptosing cells, SLPI remained in the cytoplasm. However, unstimulated HC neutrophils undergoing NETosis revealed that SLPI co-localised with the NE in the nucleus (**Fig 5.3.2.1.2.1.1, Panel 2, HC2**). The erupted neutrophil showed an overlapping pattern of all three targets (DNA, NE, and SLPI) (**Panel 1, HC2, d**). In all cases, SLPI seemed to stain with more of a grainy appearance than NE.

5.3.2.1.2.1.2. Unstimulated Non-Relapsed BD Patients' Blood Neutrophils *In Vitro*

BD patients that were considered to be clinically non-relapsed had their blood neutrophils subjected to the same culture conditions as HC subjects. Compared to HCs, there were more neutrophils attached to the uncoated, glass coverslips in 5 out of 7 (71.4%) patients' untreated cultures. In unstimulated cultures from either time point, 5 out of 7 (71.4%) non-relapsed BD patients' neutrophils also showed a clear indication that they were undergoing early NETosis (**Fig 5.3.2.1.2.1.2, yellow arrows**) by exhibiting a dense accumulation of NE (red) converging with DNA (green) within the nucleus. The same percentage of BD non-relapsed subjects' neutrophil cultures showed evidence of advanced NETosis identified by the spilling of internal contents into the extracellular environment (**Fig 5.3.2.1.2.1.2, Panel 1, BD3 and Panel 2, BD2**).

However, some neutrophils in the cultures showed a variety of unexpected morphologies which were quite different to HCs (**Fig 5.3.2.1.2.1.2, Panel 1 and 2**). There were neutrophils with large vacuoles (**Panel 1, BD2**) from one non-relapsed BD patient while another patient's neutrophils showed atypical formations with fan-like projections (**Panel 2, BD1**). No intact cells were identified in one patient's culture after 2 hrs incubation;

only cellular debris staining positive for DNA was found on the coverslips (data not shown).

During both time points, SLPI was seen to co-localise with the internal DNA/NE complex when the neutrophil was undergoing NETosis in unstimulated cultures. This is best demonstrated in **Fig 5.3.2.1.2.1.2, Panel 2, BD3**. SLPI was always found to stain within the region of externalised NETs.

SEE FIGURE 5.3.2.1.2.1.2.

Fig 5.3.2.1.2.1.2. Non-Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in an Unstimulated Culture. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were incubated for a total of 2 hrs (Panel 1) and 4 hrs (Panel 2) in culture media. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). Yellow arrows indicate definitive stages of NETosis. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.2.1.3. Unstimulated Relapsed BD Patients' Blood Neutrophils *In Vitro*

Clinically relapsed BD patients neutrophils were subjected to the same culture conditions as non-relapsed patients and HCs. **Fig 5.3.2.1.2.1.3** represents the neutrophils in unstimulated conditions in vitro originating from these relapsed BD patients (n=3). After 2 hrs in culture, 2 out of the 3 (66.7%) patients had a low number of neutrophils attaching to the coverslips (an average of 2 per frame). These cells showed evidence of early and intermediate NETosis with the convergence of NE and DNA. Their nuclear material had also decondensation and enlarged **Fig 5.3.2.1.2.1.3, Panel 1, BD1**. After 4 hrs in culture without any external chemical stimulation, both of these patient cultures showed evidence of advanced NETosis in which nearly all the cells released their contents (**Fig 5.3.2.1.2.1.3, Panel 2, BD1, BD2**).

The third patient had an increased number of neutrophils attached to the coverslips (an average of 25 cells per frame) after 2 hrs incubation. These neutrophils showed a variety of active stages: normal hypersegmented as well as early and late NETosis (**Fig 5.3.2.1.2.1.3, Panel 1, BD2**). Interestingly, after 4 hrs, the intermediate stage of NETosis (decondensed nuclei and swollen cells) predominated amongst the cells.

SLPI co-localised with the NE/DNA complex in 2 out of 3 BD relapsed patients' unstimulated neutrophil cultures in intact cells undergoing 'naturally occurring' NETosis.

SEE FIGURE 5.3.2.1.2.1.3.

Fig 5.3.2.1.2.1.3. Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in an Unstimulated Culture. Neutrophils incubated for a total of 2 hrs (Panel 1) and 4 hrs (Panel 2) in culture media. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.2.2. Unstimulated Neutrophil Cultures with NEi

5.3.2.1.2.2.1. HC Blood Neutrophils *In Vitro* with NEi

When incubating the HC neutrophils for 2 hrs with NE inhibitor (NEi), no NETosing cells were detected in any culture. The neutrophils remained hypersegmented (**Fig 5.3.2.1.2.2.1, Panel 3, HC1**) or were small, round apoptosing cells with condensed DNA and cytoplasmic contents (**Panel 3, HC2**). A few bi-lobed eosinophils (SLPI⁺/NE⁻) were also detected. After 4 hrs in culture, despite the NEi, a few neutrophils did progress to early (**Panel 4, HC1**) and intermediate NETosis (**HC2**).

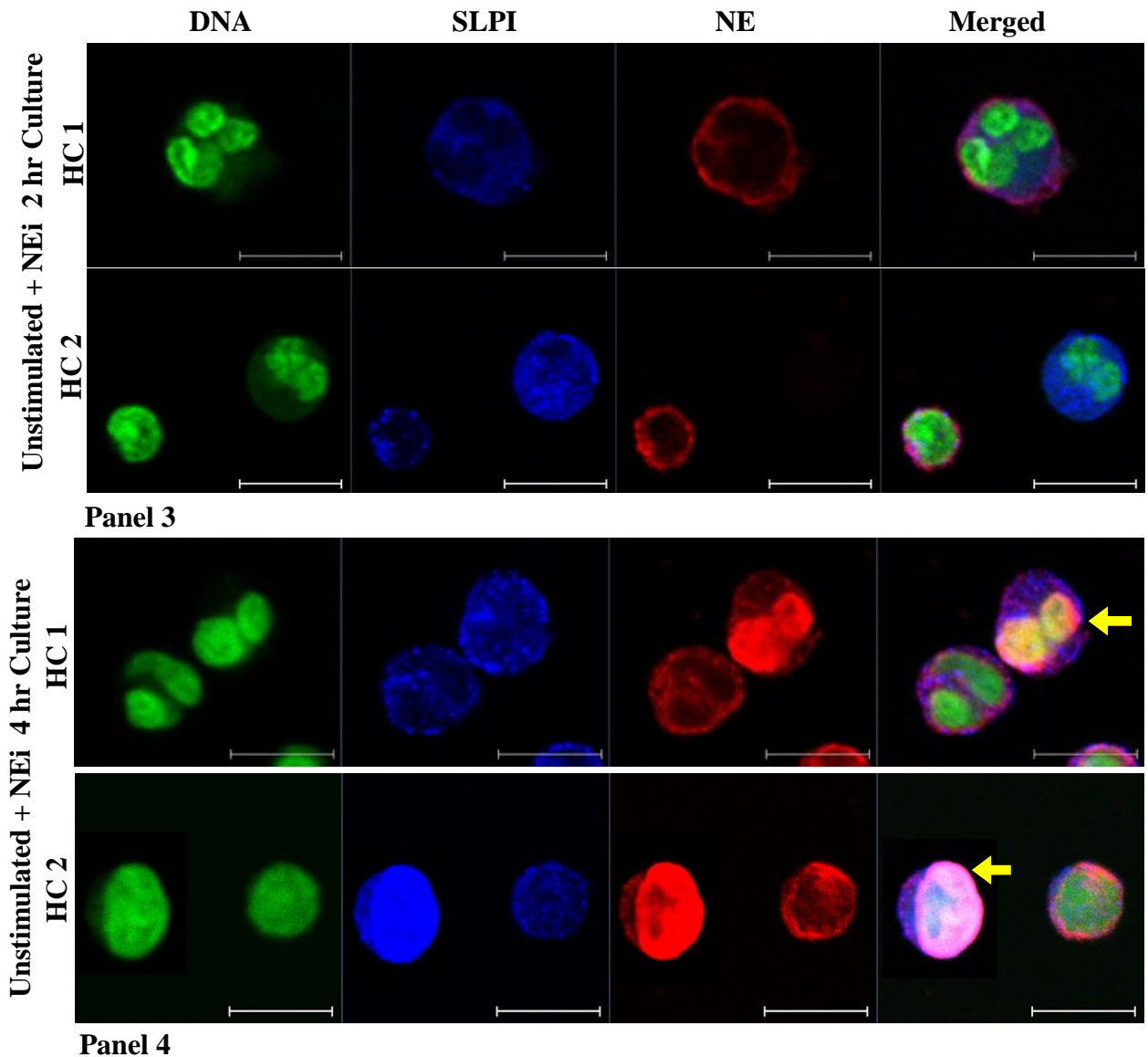


Fig 5.3.2.1.2.2.1. Unstimulated HC Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in Unstimulated Cultures with NE Inhibitor. Neutrophils were incubated for a total of 2 hrs (Panel 3) and 4 hrs (Panel 4) in culture media with NEi before being fixed in 4% PFA. The cells were stained for NE (red), SLPI (blue), and DNA (DAPI) (green). Co-localisation of all three targets resulted as pink areas. Yellow arrows indicate various stages of NETosis. X40 oil magnification, Confocal 710. Bar indicates 10 μm.

5.3.2.1.2.2.2. Non-Relapsed BD Patients' Blood Neutrophils *In Vitro* with NEi

With the addition of 5 μ M of NEi during the 2 hr incubation, 4 out of 7 (57.1%) BD non-relapsed patients' neutrophils showed fewer cells undergoing NETosis (**Fig 5.3.2.1.2.2.2, Panel 3, BD1**, arrow) compared to their unstimulated counterpart without NEi. After 4 hrs, 6 out of 7 (85.7%) cultures did not show any signs of NETosing cells except for BD3 in **Fig 5.3.2.1.2.2.2, Panel 4** (arrow).

Some of the other cell characteristics that were detected in cultures with NEi are represented in **Fig 5.3.2.1.2.2.2, Panel 3 and 4**. Eight out of 14 (57.1%) cultures regardless of their incubation time of 2 or 4 hrs showed some normal, hypersegmented nuclei (**Panel 3: BD2; Panel 4: BD1, BD2**). However, similar to the unstimulated cultures, some neutrophils had an atypical morphology. For instance, the cell in **Panel 3** from **BD1** had decondensed DNA and blebbing without co-localising NE. There were cells that seemed to be spilling their contents (**Panel 3, BD2**) and neutrophils thought to be undergoing apoptosis (**Panel 3, BD3**).

SEE FIGURE 5.3.2.1.2.2.2

Fig 5.3.2.1.2.2.2. Non-Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in an Unstimulated Culture with NE Inhibitor. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were incubated for a total of 2 hrs (Panel 3) and 4 hrs (Panel 4) in culture media with NEi. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). Yellow arrows indicate definitive stages of NETosis. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.2.2.3. Relapsed BD Patients' Blood Neutrophils *In Vitro* with NEi

The addition of NEi dramatically reduced the occurrence of NETosis in the BD relapsed patients' neutrophils. Two out of the 3 patients whose cells showed evidence of NETosis in their unstimulated cultures, now showed normal, hypersegmented neutrophils after 2 hrs incubation (**Fig 5.3.2.1.2.2.3, Panel 3, BD1**). Only one patients showed evidence of NETosing cells in the presence of NEi (**Panel 3, BD2**). Following the 4 hr incubation with NEi, none of the 3 patients' neutrophils had progressed toward cell death via the NETosis pathway (**Panel 4, BD1, BD2**). Neutrophils with this normal morphology retained NE and SLPI in the cytoplasm.

SEE FIGURE 5.3.2.1.2.2.3.

Fig 5.3.2.1.2.2.3. Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in an Unstimulated Culture with NE Inhibitor. Neutrophils were incubated for a total of 2 hrs (Panel 1) and 4 hrs (Panel 2) with NEi in culture media. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.2.3. PMA-Stimulated Neutrophil Cultures

5.3.2.1.2.3.1. PMA-Stimulated HC Blood Neutrophils *In Vitro*

The next experiment was to use PMA to induce NETosis in HC neutrophils as a positive control. The results are shown in **Fig 5.3.2.1.2.3.1**. The most obvious observation was that the activated neutrophils had readily attached to the glass coverslips. The neutrophils from 3 out of 4 (75%) HC subjects reacted to the PMA after 1 hr of stimulation (**Panel 5, HC1**). Only 1 HC subject's cells remained unaffected (**Panel 5, HC2**). After 3 hrs of PMA-stimulation (and a total of 4 hrs in culture) (**5.3.2.1.2.3.1, Panel 6**), many of the HC subjects' neutrophils had reacted with a few showing the end stage of NETosis in which the cell contents were cast out into the local environment (**Panel 6, HC1**). SLPI was not seen to co-localise with the NE/DNA complex in PMA-induced HC neutrophils until expelled from the cell in a NET. In all the cultures, the eosinophils, identified by their SLPI⁺/NE⁻ staining and bi-lobed nucleus, were never seen to undergo NETosis with or without PMA-stimulation.

SEE FIGURE 5.3.2.1.2.3.1.

Fig 5.3.2.1.2.3.1. HC Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs (Panel 5) and 4 hrs (Panel 6) in culture media without NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). Yellow arrows indicate various stages of NETosis. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.2.3.2. PMA-Stimulated Non-Relapsed BD Patients' Blood Neutrophils *In Vitro*

When non-relapsed BD patient neutrophils were exposed to PMA in culture, within 1 hr, 4 out of 7 (57.1%) samples showed evidence of advanced progression of NETosis. The classic characteristics included the expulsion of cell contents which is demonstrated in **Fig 5.3.2.1.2.3.2, Panel 5, BD3**. In fact, two of these samples only showed cellular debris left on the coverslip. Two out of 7 (28.6%) samples showed the convergence of NE with DNA and the beginning of nuclear decondensation indicative of early NETosis (**Panel 5, BD2**). Only one sample had not yet reacted to the PMA (**Panel 5, BD1**).

After 3 hrs of PMA-stimulation (a total of 4 hrs in culture), all 7 (100%) non-relapsed BD subjects' cultures had reacted to the stimulant. All the cultures showed intermediate or advanced NETosis. The intermediate stage of NETosis was observed in 4 out of 7 (57.1%) cultures including cytoplasm and nuclear vacuolization as well as cell swelling and decondensing of nuclear DNA (**Fig 5.3.2.1.2.3.2, Panel 6, BD1**). The casting out of cellular contents is the final act in advanced NETosis. This was observed in 3 out of the remaining 7 cultures (**Panel 6, BD2, BD3**).

SEE FIGURE 5.3.2.1.2.3.2.

Fig 5.3.2.1.2.3.2. Non-Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs (Panel 5) and 4 hrs (Panel 6) in culture media without NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.2.3.3. PMA-Stimulated Relapsed BD Patients' Blood Neutrophils *In Vitro*

When stimulating the relapsed BD patients' neutrophils with 100 nM of PMA, all of the cells in culture reacted after only 1 hr of exposure. One patients' neutrophils showed decondensation of the nuclear material and swelling indicative of the intermediate stage of NETosis (**Fig 5.3.2.1.2.3.3, Panel 5, BD1**). Meanwhile, the other two patients' cell cultures were undergoing advanced NETosis with the expulsion of cell contents into the surrounding environment as seen in **Fig 5.3.2.1.2.3.3, Panel 5, BD2**. After 3 hrs of PMA-stimulation, all the cells from all 3 relapsed BD patients had undergone advanced NETosis (**Panel 6**).

SLPI was not observed to co-localise with the NE/DNA complex in intact, PMA-induced neutrophils but this was probably because most of the cells' contents had already been expelled. Among the NETs, SLPI stained in a similar but not exact pattern of the externalised NE and DNA.

SEE FIGURE 5.3.2.1.2.3.3.

Fig 5.3.2.1.2.3.3. Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture. Neutrophils were incubated for a total of 2 hrs (Panel 5) and 4 hrs (Panel 6) in culture media without NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.2.4. PMA-Stimulated Neutrophil Cultures with NEi

5.3.2.1.2.4.1. PMA-Stimulated HC Blood Neutrophils *In Vitro* with NEi

Despite the addition of NEi, most PMA-stimulated HC neutrophils continued to undergo early NETosis as indicated by the arrows in **Fig 5.3.2.1.2.4.1, Panel 7** and **8**, however, advanced NETosis was observed in only one HC culture. One other culture showed no progression towards NETosis at all (**Panel 7, HC2**); NE remained separated from the DNA. Therefore, NEi probably inhibited or delayed these HC neutrophils from undergoing NETosis.

SEE FIGURE 5.3.2.1.2.4.1.

Fig 5.3.2.1.2.4.1. HC Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture with NE Inhibitor. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs (Panel 7) and 4 hrs (Panel 8) in culture media with NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). Yellow arrows indicate various stages of NETosis. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.2.4.2. PMA-Stimulated Non-Relapsed BD Patients' Blood Neutrophils *In Vitro* with NEi

Incorporating 5 μ M of NEi before the PMA was added to the cultures seemed to hinder progression of induced NETosis in a total of 9 out of 14 cultures (64.3%): 3 out of 7 (42.9%) cultures after 1 hr of PMA-stimulation and 5 out of 7 (71.4%) cultures after 3 hrs of stimulation. Cultures showed a mix of hypersegmented, normal neutrophils as well as cells undergoing the convergence of their NE into the DNA (**Fig 5.3.2.1.2.4.2, Panel 7, BD1**) signifying early NETosis which was still apparent after 3 hrs of PMA-stimulation (**Panel 8**). SLPI was not observed to co-localise with the NE/DNA complex while the PMA-stimulated, BD non-relapsed patient neutrophils remained intact.

SEE FIGURE 5.3.2.1.2.4.2.

Fig 5.3.2.1.2.4.2. Non-Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture with NE Inhibitor. Neutrophils were incubated for a total of 2 hrs (Panel 7) and 4 hrs (Panel 8) in culture media with NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.2.4.3. PMA-Stimulated Relapsed BD Patients' Blood Neutrophils *In Vitro* with NEi

Despite NEi in the culture, an hour of PMA-stimulation showed that neutrophils from 2 out of the 3 BD relapsed patients underwent advanced NETosis (**Fig 5.3.2.1.2.4.3, Panel 7, BD2**). This remained evident after 3 hrs (**Panel 8, BD2**). However, it was worth noting that some of the cells in these cultures still had DNA that was lobular without NE and DNA mixing. Also after 3 hrs of PMA exposure, the third relapsed BD patient whose neutrophils retained normal, hypersegmented nuclei after 1 hour's stimulation began to show cellular swelling and DNA decondensation (**Panel 8, BD1**).

SEE FIGURE 5.3.2.1.2.4.3.

Fig 5.3.2.1.2.4.3. Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture with NEi. Neutrophils were incubated for 2 hrs (Panel 7) and 4 hrs (Panel 8) in culture media with NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

Despite 1 hr of PMA-stimulation, by including NEi in the initial culture, one of the relapsed BD patients' neutrophils was able to retain normal morphology (**Fig XIII, Panel 7, BD1**). Previously, with PMA alone, this patient showed intermediate stage of NETosis. NEi was able to inhibit NE from converging with the DNA and thus preserving the hypersegmentation in the relapsed BD patients' cultures.

5.3.2.1.2.5. Unidentified Cell Death

A few of the unstimulated cultures had a small selection of neutrophils that were undergoing cell death, but the type of process could not be identified solely with the protein targets that were used. A selection of these cells is demonstrated in **Fig 5.3.2.1.2.5.1**. The top panel shows a HC neutrophil in which the nuclear DNA has been disrupted and fills up the entire space within the cell's borders. However it maintains a compact, round morphology (possibly attributed to apoptosis) with NE on the periphery while SLPI is distributed throughout the cell. The middle panel shows a BD patient's neutrophil whose nucleus was still hypersegmented with some infiltration of NE indicative of NETosis, but seemed as if its cytoplasm ruptured before the process could finish. The last panel again shows a neutrophil from a different BD patient. The DNA was broken down and had expanded through-out the cell. SLPI had a similar distribution contained within an irregular cell border. A small patch of NE existed in the cell centre. The expansion of the cell larger than 10 μm could be a sign of secondary necrosis.

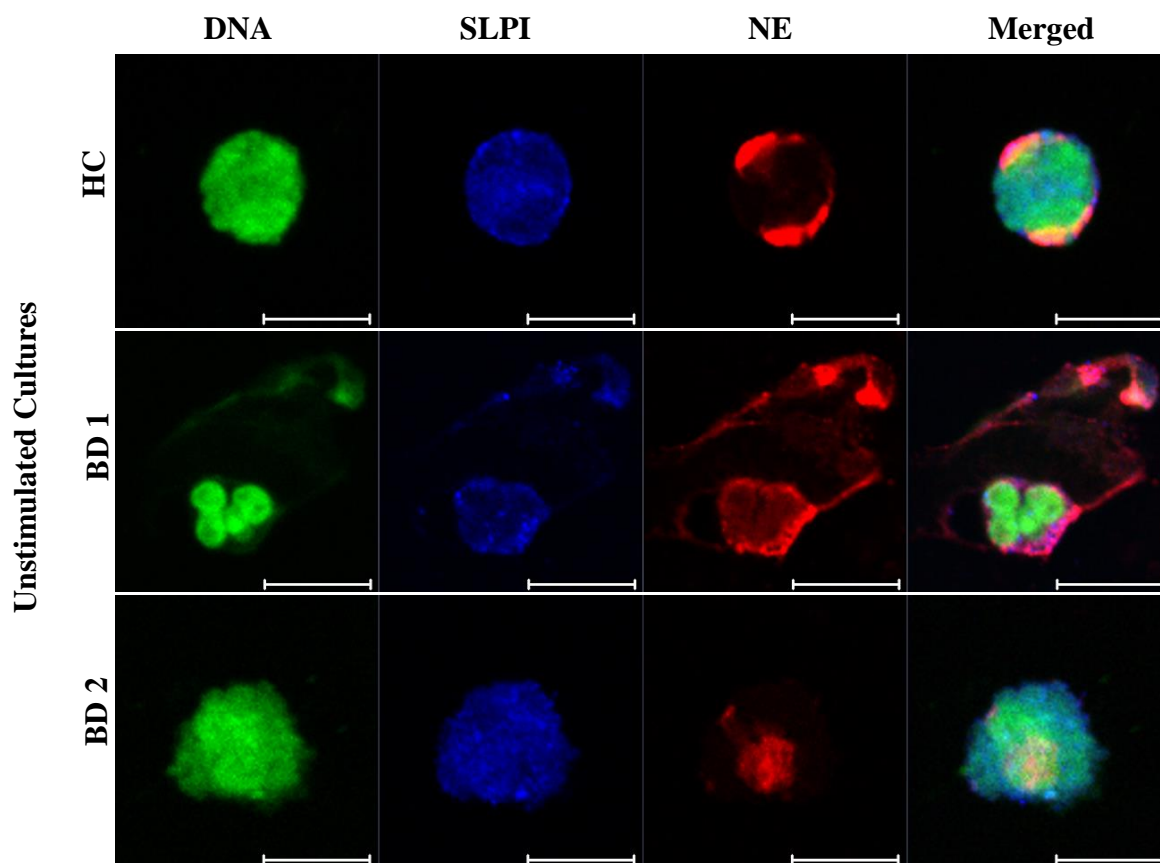


Fig 5.3.2.1.2.5.1. HC and Non-Relapsed BD Patients' Neutrophils *In Vitro*: Undefined Cell Death Processes in Unstimulated Cultures. Neutrophils were incubated for 2 and 4 hrs in culture media. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μm.

5.3.2.1.3. MPO and NETosis

After NE's movement to the nucleus, its azurophil co-inhabitor, MPO, is selectively translocated from the granule to the nucleus during NETosis (Papayannopoulos et al., 2010). To observe this event, the cultured, fixed neutrophils from all patient groups

Fig 5.3.2.1.3.1. Positive Control: Neutrophils Undergoing NETosis in a PMA-Stimulated Culture. Demonstrated in the panels above are the typical morphologies of NETosing HC and BD neutrophils when they were induced with PMA. The main MPO activity occurred in the intermediate stage of NETosis after NE had translocated into the nucleus, therefore the first image shows this integration of MPO (blue) into the nucleus. The second and third panels show progressed intermediate stages in which MPO granules in the cytoplasm become more concentrated and pronounced, decondensation of DNA (green) takes place, and the cell continues to swell and vacuoles may develop both in the cytoplasm and nucleus. The final panel shows the advanced stage of NETosis in which cells erupts and casts out NETs. The asterisk marked panels signify the commonly observed progression of NETosis from decondensed nuclei to advanced NETosis. Confocal 710, X40 oil magnification. Bar indicated 10 μ m.

SEE FIGURE 5.3.2.1.3.2.

Fig. 5.3.2.1.3.2. Unstimulated and PMA-Stimulated HC Neutrophils *In Vitro*: Observation of MPO. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs and 4 hrs in culture media before being fixed in 4% PFA. The cells were stained for NE (red), MPO (blue) and DNA (green). Co-localisation of all three targets produced pink regions. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

SEE FIGURE 5.3.2.1.3.3.

Fig. 5.3.2.1.3.3. Unstimulated and PMA-Stimulated Non-Relapsed BD Neutrophils *In Vitro*: Observation of MPO. Neutrophils were incubated for a total of 2 and 4 hrs in culture media before being fixed in 4% PFA. The cells were stained for NE (red), MPO (blue) and DNA (green). Co-localisation of all three targets produced pink regions. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

SEE FIGURE 5.3.2.1.3.4.

Fig 5.3.2.1.3.4. Unstimulated and PMA-Stimulated Relapsed BD Neutrophils *In Vitro*: Observation of MPO. Neutrophils were incubated for a total of 2 and 4 hrs in culture media before being fixed in 4% PFA. The cells were stained for NE (red), MPO (blue) and DNA (green). Co-localisation of all three targets produced pink regions. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

5.3.2.1.4. The Role of Endogenous Alpha-1 Antitrypsin During NETosis

Neutrophils also harbour another endogenous NE inhibitor, alpha-1 antitrypsin (α 1AT), which is stored in the azurophil granules alongside NE (Mason et al., 1991). The cultured PFA-fixed neutrophils were subjected to staining with NE and α 1AT antibodies in order to

observe if co-localisation occurred between serine protease and serpin inhibitor intracellularly while undergoing NETosis or after the event of casting out.

In **Fig 5.3.2.1.4.1**, unstimulated HC neutrophils stained positive for NE, α 1AT, and DNA. The neutrophils underwent early NETosis as NE began to mix with the DNA acting to deconstruct its histone and chromatin assembly. A higher concentration of NE can be seen on the periphery of the lobed nuclei which can be seen in the first panel. Meanwhile, the α 1AT (in purple) seems to remain in spotted granules throughout the cytoplasm. There does not seem to be any overlap of α 1AT with either the NE or diffused DNA staining. To compare α 1AT's cytoplasmic location in HCs, additional staining was completed on BD patients' neutrophils for all the experimental culture conditions including unstimulated and PMA-stimulated NETosis with or without NEi. A representative panel of the results can be seen in **Fig 5.3.2.1.4.1**.

SEE FIGURE 5.3.2.1.4.1.

Fig 5.3.2.1.4.1. Alpha-1Antitrypsin during NETosis *In Vitro*. HC, BD non-relapsed and relapsed patients' neutrophils were incubated with and without PMA then PFA-fixed and stained for NE (red), α 1AT (purple), DNA (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

As seen previously, NE co-localised with DNA during all stages of NETosis regardless of donor or culture treatment (with or without PMA). However, the α 1AT appeared exclusively within the cytoplasm – presumably still packaged in the azurophil granules. It was only upon the release of NETs that α 1AT would speckle-coat the area around NE and DNA as seen in **Fig 5.3.2.1.4.1**. The addition of NEi did not seem to have any effect on

changing the location of α 1AT in the cytoplasm. This was evident in both unstimulated and induced NETosis with NEi (data not shown).

5.3.3. Summary of Key Results

The key results from the immunofluorescent staining of fixed neutrophils from HC and BD patient cultures are listed in the **Table 5.3.3.1.** below.

Key Results	Number of Patients (%) in at Least 1 Culture Time Point
Advanced NETosis occurring in unstimulated cultures	2/4 (50%) HC 5/7 (71.4%) BD Non-Relapsed* 3/3 (100%) BD Relapsed*
NEi reduced the progression of NETosis compared to unstimulated cultures without NEi at the same time point*	3/3 (100%) HC 4/7 (57.1 %) BD Non-Relapsed 2/3 (66.7%) BD Relapsed
SLPI but not α 1AT was seen to co-localise with NE/DNA in intact cells in unstimulated cultures	2/3 (66.7%) HC 5/7 (71.4%) BD Non-Relapsed 2/3 (66.7%) BD Relapsed
Advanced NETosis occurring after 1 hr of PMA-stimulation*	1/4 (25%) HC 3/7 (42.9%) BD Non-Relapsed 2/3 (66.7%) BD Relapsed

Table 5.3.3.1. Summary of Key Results *based on SLPI/NE staining data

Differences	Similarities
--------------------	---------------------

<ul style="list-style-type: none"> • Neutrophils did not swell in unstimulated cultures as much as with PMA • Intact cells were 9-10 μm in diameter whereas PMA-stimulated neutrophils could be seen swelling in excess of 15 μm • In unstimulated cultures, decondensed DNA did not enlarge as much as PMA-induced cells • Vacuoles were rarely seen in cells undergoing NETosis in unstimulated cultures (only seen in 1 BD patient) • SLPI co-localised with the NE/DNA complex in the nuclear region of intact neutrophils in unstimulated cultures but not when PMA was included • Unique morphologies with distinct DNA/NE/SLPI-coated extracellular fibres seen in BD patients <ul style="list-style-type: none"> ➤ was repressed by NEi • In most cases, the number neutrophils that adhered to coverslips increased when exposed to PMA 	<ul style="list-style-type: none"> • NE re-located to the nucleus and co-localised with DNA • MPO entered and co-localised with the NE and DNA at a late-stage of NETosis • NEi could inhibit the progression of NETosis • The released NET DNA fibres were coated with NE, MPO, SLPI, and $\alpha 1\text{AT}$ • When externalised, DNA, NE, MPO, SLPI, and $\alpha 1\text{AT}$ adhered to the glass coverslips
---	--

Table 5.3.3.2. Differences and Similarities Collectively Observed When Comparing PMA-Stimulated and Non-Stimulated Neutrophils from All Patient Groups *In Vitro*.

5.4. Discussion

5.4.1. Challenges of Working with Neutrophils in the Lab

5.4.1.1. Time Schedule Constraints

Neutrophils in the peripheral circulation live a relatively short time compared to other leukocytes (Hallett, 1997, Webb et al., 2000). They are terminally senescent cells and therefore will not replicate. When conducting experiments with *ex vivo* human neutrophils, time is constrained. The *in vitro* experimental treatments and sytox[®] green NETs quantification conducted for this study had to be completed in one day. The cells cultured on glass coverslips were able to be PFA-fixed and immunofluorescent stained at a later date.

5.4.1.2. Avoiding Neutrophil Priming or Activation

One of the most vital issues with assessing NETs from human blood was the possible activation of the neutrophils during purification and handling. Neutrophils exist in the peripheral blood in three states: quiescent, primed, or active (Hallett and Lloyds, 1995). A primed neutrophil differs from an active one because it has recognised a stimulus via a cell receptor but has not yet triggered an oxidase response (Hallett and Lloyds, 1995). Neutrophils can generate a superoxide respiratory burst upon adherence to plastics (Ginis and Tauber, 1990) or with other protocol reagents such as gelatin (Stie and Jesaitis, 2007). We were aware that these may potentially create a situation where the risk of generating false positive results was increased. Due to this potential limitation, blood from HCs underwent the same procedures as BD patients' blood and had their production of NETs assessed under the same conditions in parallel.

Several difficulties were overcome during neutrophils isolation. Firstly, the Histopaque 1119 density separation reagent and blood samples had to be at RT otherwise the blood did not filter through the gradient to isolate red blood cells from PBMC's and granulocytes. Several patients' blood samples were unfortunately unusable for downstream analysis due to the inability to filter properly. Also, if the donor was anaemic, the blood would not separate properly which was the case with one HC where only a few cultures could be prepared due to the poor neutrophil isolation. Secondly, it was found that Brinkmann, *et al*, (Brinkmann et al., 2004) description of percoll gradients was misleading. In their published protocol, the first step in diluting the neat percoll was to create a 90% solution in PBS. However, confusingly, they refer to this first dilution as "100%" solution and instruct the reader to create a 65, 70, 75, 80 and 85% solution from it, but is actually 58.5, 63, 67.5, 72, and 76.5%, respectively. This was initially misconstrued and could have been better described. Also, the use of RBC lysis solution had been included by nearly all previous NET protocols (Skrzeczynska-Moncznik et al.,

2012, O'Donoghue et al., 2013), but there were warnings about this procedure because of the high risk of activating the neutrophils and so RBC lysis reagents were avoided.

5.4.1.3. Neutrophil Adherence to Non-Coated Glass Coverslips

Glass coverslips were initially coated with poly-D-lysine, however it was later discovered that the synthetic polymer may also have activated the neutrophils and disrupted the DNA making NETs. Poly-D-Lysine, a polyamino acid, increases the electrostatic interaction and binding between cations on surface materials used for culturing and cell membrane anions. A high molecular weight (150-300k) poly-D-lysine was chosen for these experiments in order to encourage neutrophils to attach to the glass coverslips as neutrophils are notoriously difficult to attach without stimulation. Whilst we do not know for certain how poly-D-lysine triggered NET formation, it could be due to the interaction of negatively charged extracellular DNA and positively charged polymer.

Culturing neutrophils on non-coated glass coverslips had its challenges. A large number of neutrophils (1×10^5 - 5×10^5 cells per well) were required for the cultures destined for PFA-fixing and antibody staining due to repetitive wash procedures which depleted the number of neutrophils as they easily lifted off of the non-coated coverslips. This was the case especially with unstimulated cultures from HC and two BD non-relapsed patients. It also became apparent that non-stimulated neutrophils showed a reduced ability to adhere to the glass coverslips. Previous studies have shown similar occurrences when culturing human neutrophils for purposes other than observing NETs. Smith, *et al*, (Smith et al., 1979) exposed HC neutrophils *in vitro* to various chemotactic factors including zymosan yeast activated serum, *E.coli* bacterial extract, the proinflammatory complement fragment C5a, and synthetic peptide N-formyl-L-methionyl-L-phenylalanine (FMLP). They found that each chemotactic factors tested caused a significant increase in the number of neutrophils that were able to attach to glass compared to those without any chemotactic factors exposure. The cell shape was altered from spherical to polarised (bipolar) as well. They also showed that pre-coating the glass with BSA or 5% serum reduced the attachment of round-shaped neutrophils but not of polarised neutrophils. Webster, *et al*, (Webster et al., 1986) found that PMA, a non-chemotactic stimulus, also increased neutrophil adherence in a concentration dependent manner. In our study, we also found that PMA-stimulated neutrophils attached readily to the coverslips. Therefore, neutrophils that were not PMA-stimulated but were potentially exposed to circulating chemotactic factors *in vivo* (as is possible in BD patients) may have promoted cell attachment *in vitro*.

Lastly, very few intact, non-stimulated neutrophils could be located on the slides when NEi was added to unstimulated cultures. NEi inhibits NE activity and as a consequence appeared also to have reduced the neutrophils' adhesion to the coverslips.

5.4.1.4. The Different Morphology of NETs Created by Laboratory Handling

When neutrophils were cultured in order to determine whether they are undergoing NETosis, we found that there were two morphological states that the neutrophil could be visualised depending on the operator's handling and manipulation. First, if they were cultured on glass coverslips, stained with sytox and then lifted, inverted and mounted onto slides, the neutrophils which underwent NETosis showed vivid and characteristic DNA net-like protrusions. This formation made it difficult to accurately quantify the number of NETosing cells. The second option was to culture the cells directly onto semi-adherent culture wells, add sytox green to the media, and view them under an inverted timelapse fluorescent microscope with minimum mechanical disruption to the cells as previously described by Papayannopoulos, *et al*, (Papayannopoulos et al., 2010) and Brinkmann and Zychlinsky (Brinkmann et al., 2012). This technique provided a clearer image in order to determine the number of NETosing cells. Granted, it can be argued that a stationary culture of neutrophils does not necessarily reflect their non-static nature *in vivo*, but for the purpose of initially determining the quantification of the cells, the stationary culture is currently the best option.

5.4.1.5. Immediate Imaging is Necessary for Accurate Sytox Quantification

Imaging the same unstimulated HC neutrophil cultures the next day resulted in a small, non-significant increase in sytox⁺ cells. However, when re-imaging PMA-stimulated HC cells, there was a dramatic increase in sytox⁺ cells the next day. This is most likely due to the prolonged chemical stimulation in culture overnight. Therefore, imaging was carried out for all sytox experiments.

5.4.1.6. Quantification of NETs

Quantifying extracellular DNA from NETs using sytox[®] green and Hoechst provided the first evidence that BD patients had a higher incidence of NETosing cells than HCs. In particular, the average percent of sytox⁺ neutrophils were from BD patients experiencing a clinical relapse. Further analysis revealed the increase of sytox⁺ cells after 4 hrs in unstimulated cultures from BD patients but not HCs. This data suggests that BD patients' neutrophils were producing more NETs than HCs. BD relapse patients' neutrophils also produced more sytox⁺ cells after 3 hrs of incubation with PMA than either BD non-

relapsed patients or HCs further supporting our hypothesis that their cells had been primed or activated by an unknown factor *in vivo*.

5.4.2. HC and Behçet's Disease Patients NETs

We have shown that blood neutrophils from HC and BD patients are capable of producing NETs in standard culture conditions without the addition of an exogenous chemical stimulant or pathogen. However, NETs were more abundant in BD patients' unstimulated cultures, especially if the individuals were clinically relapsed. This evidence suggests that BD patients harbour a high number of primed or activated circulating neutrophils and there may be an activating factor in the peripheral blood inducing NETosis. We also found that the longer the neutrophils from BD relapsed patients were incubated, the more cells were seen to be undergoing NETosis compared to HCs. Interestingly, the components that were targeted – NE, MPO, SLPI, α 1AT, and DNA – showed similar staining patterns in both unstimulated neutrophil cultures from HC and BD patients. However, in some BD patients cultures, the cell morphology was unique exhibiting fan-like projections of the cell membrane with co-localised NE, DNA, and some SLPI whilst still having polymorph nuclei.

Similar to Papayannopoulo's, *et al*, findings, in our PMA-stimulated experiments NE was detected both in the cytoplasm and with the nuclear DNA (Papayannopoulos et al., 2010). However, during NETosis in both HC and BD patients' unstimulated cultures, NE was not often observed in the cytoplasm. If cytoplasmic NE was observed, its intensely staining was low. This suggests that the translocation of NE induced by PMA may be via a different mechanism from that which occurs in unstimulated HC and BD neutrophils. In addition, the PMA pathway may require a lower internal concentration of NE to destabilise the chromatin and therefore NE granules can still be detected in the cytoplasm. When targeting MPO, it was observed in the cytoplasm at the late-stage of NETosis when the nucleus had already decondensed and lost its lobular formation, but with the cell membrane still intact. It has been reported that in PMA-stimulated neutrophils, MPO is transported to the nucleus separately and at a later time point than NE (Papayannopoulos et al., 2010) even though they are stored in the same granule. We observed the same occurrence in both PMA and non-stimulated cultures from both HC and BD groups. This suggests that MPO has a similar role during NETosis under these conditions.

Individuals with the immunodeficiency condition CGD suffer recurrent infections due to their inability to produce ROS and are defective in making NETs (Fuchs et al., 2007). BD patients, on the other hand, suffer from a hyperimmune state in which an increased

number of active neutrophils are circulating (Matsumura and Mizushima, 1975, Verity et al., 2003), and according to our sytox[®] green quantification and immunofluorescence imaging data, producing NETs. However, this profusion of NETs in BD may be detrimental and contribute to an over-reactivity to foreign or self-antigens.

A study conducted by Nakahara, *et al.*, in 1998 showed that HC oral polymorphonuclear leukocytes are primed and spontaneously generate a slew of ROS by an unknown superoxide dismutase sensitive mechanism releasing MPO (Nakahara et al., 1998). They may have been describing NETosis in oral neutrophils before it was formally identified as a cell-death pathway. To date, no experiments have been conducted on the occurrence of NETs in saliva although they have been identified in CF sputum (Papayannopoulos et al., 2011). NETs have also been identified in cultured neutrophils from bronchoalveolar lavage fluid after treating mice lungs with LPS (Saffarzadeh et al., 2012). Therefore, it can be speculated that neutrophils in the upper airway and oral cavity that are perpetually exposed to various microbes probably have a high incidence of NETs.

Streptococcus sanguinis bacteria has been shown to have an increased association with the oral flora of BD patients (Hatemi and Yazici, 2011). Several studies have also found that poor periodontal indices and overall oral health are associated with disease severity (Akman et al., 2007, Arabaci et al., 2009, Mumcu, 2004). Oral ulcers also correlated with an impaired periodontal state (Mumcu, 2004). Recently, Vitkov, *et al.*, found abundant NETs in purulent exudate from the gingival crevice of those with periodontitis (Vitkov et al., 2009). It is conceivable that BD patients, especially those with active ulcers, would have a high number of NETosing neutrophils in their oral cavity. This would also help to explain the high levels of NE we found in their saliva (**Section 4.3.2.1**).

BD patients also have a continuously high levels of circulating IL-8 (Durmazlar et al., 2009) which our data in **Chapter 3.3.1** confirmed in saliva. It has been demonstrated that at high IL-8 concentrations neutrophil azurophil degranulation occurs (Ley, 2002) (Baggiolini et al., 1989). IL-8 has also been shown to induce NETosis (Brinkmann et al., 2004, Gupta et al., 2005) although there is some debate whether IL-8 can prime the neutrophil but cannot trigger NETs (Branzk, 2013). The increased levels of IL-8 in BD patients could therefore be driving neutrophils towards the NETosis cell-death pathway instead of apoptosis.

The release of NETs in the vasculature could be contributing to the inflammation and pathology of BD. Recently, Fuchs, *et al*, confirmed that when stimulated neutrophils created NETs, platelets and RBCs accumulated and aggregated on the DNA/histone fibres (Fuchs et al., 2010). Consequently, the scaffolding of a thrombus formed which was demonstrated in 3D flow chambers. Fibrinogen, fibronectin, and von Willebrand factor were also immobilised on the NETs. *In vitro*, NETs were able to be dismantled with DNase and heparin (Fuchs et al., 2010). About 40% of the individuals with BD have vascular involvement of various forms including deep vein thrombosis and aneurysms (Seyahi and Yurdakul, 2011). NETs may be contributing to these events.

As mentioned previously, NETs induced by PMA *in vitro* are inhibited by high serum content (Fuchs et al., 2007). In a normal human circulatory system, plasma (which has clotting factors) makes up 55% of the blood, therefore in theory should reduce the quantity of NETs produced *in vivo* with the exception of those in tissue. Also, extracellular chromatin from necrotic cells is usually degraded by serum endonucleases such as DNase I (Napirei et al., 2004) and HC serum has been shown to degrade NETs (Hakim et al., 2010).

A recent study demonstrated the cytotoxic damage NETs can cause by culturing NET-derived components with A549 human lung epithelial cells as well as pulmonary artery and umbilical vein endothelial cells (Saffarzadeh et al., 2012). They PMA-stimulated HC neutrophils *in vitro* for 4 hrs, washed the cells, agitated the culture to release the NET material and after a very gentle centrifugation, collected the supernatant containing the NET material. This was then treated with micrococcal nuclease (MNase), DNase, or left undigested. They showed that the cytotoxicity of NET material on A549 cells (based on decreased confluence and increased multicaspase activity) increased with all 3 treatments. The pulmonary artery and umbilical vein endothelial cell cultures also showed similar results. Therefore, the authors concluded that DNA was not responsible for altering cell growth or cell death and that purified histones and MPO were partially responsible for cytotoxic effects. They also showed that NE was active in the NET material and increased significantly when the DNA-component in NETs was digested with DNase or MNase. This suggests that DNA-bound NE partially reduces NE activity, but when NE is released from the bond DNA, ensues increased activity. Even though A549 lung epithelial cells are known to produce both SLPI and α 1AT (Sallenave et al., 1994, Venembre et al., 1994), the cytotoxicity to A549 cells remained high despite the use of NE inhibitor. This

lead to the belief that damage to the cells is not solely due to NE. These are progressive and important findings of the damage NETs can cause. However, it is important to note that PMA was used as the stimulant in these experiments and since the NETosis mechanism seems to differ depending on the stimulant as alluded to by Parker, *et al*, (Parker et al., 2012), it could have affected the components bound to the NETs.

The various periodic symptoms that are endured by BD patients include joint inflammation and pain similar to arthritis. In a comparison to psoriatic arthritis, BD synovial fluid and biopsies have a higher number of neutrophils despite having similar IL-8 levels (Canete et al., 2009). Although NETs have not been investigated in BD joints, people with Rheumatoid Arthritis (RA) have also been found to harbour NETs in their synovial fluid (Khandpur et al., 2013). A positive pathergy test is included as part of the diagnostic criteria, although 100% of BD do not have a positive result, however histologically skin biopsies show infiltrating neutrophils and T-lymphocytes (Ergun et al., 1998). Recently, NETs have been detected in psoriatic skin biopsies (Skrzeczynska-Moncznik et al., 2012). It would be helpful to conduct further investigations of any NETs involvement with BD skin.

5.4.2.1. BD Patients' Neutrophils React Quickly to PMA Producing Enlarged Cells and Expansive NETs

NETs result when PMA activates the NADPH oxidase enzyme complex at cell and phagosomal membranes by directly stimulating the enzyme protein kinase C- β isotype (Gray et al., 2013, Dekker et al., 2000, Brinkmann and Zychlinsky, 2012). Although PMA is synthetic and not produced *de novo*, it was chosen due to its ability to produce large quantities of superoxide and H₂O₂ (DeChatelet et al., 1976) and has been the main stimulant employed for the majority of published NET experiments. PMA has been described as producing a similar effect to phagocytosis (DeChatelet et al., 1976). This was confirmed by Takei, *et al*, (Takei et al., 1996) who showed that PMA-induced neutrophils could still phagocytose opsonised particles.

We found that BD patients' neutrophils were more susceptible to producing NETs after stimulation with PMA than HCs. In the majority of BD patient cultures, NETs were readily observed after only 1 hour with PMA-stimulation. The effect of PMA on HC neutrophils was not as potent. When induced, not all HC cells in the culture were affected; many retained the typical neutrophil features: condensed, multi-lobed nucleus

and a rounded cell less than or equal to 10 μm in diameter. Intact, PMA-stimulated neutrophils were nearly double the size of the cells undergoing NETosis in unstimulated cultures and resulted in a large expanse of NETs. This was true for stimulated neutrophils from both HC and BD patients.

BD patients' neutrophils showed this prompt reaction to PMA probably due to their hyperimmune state (Eksioglu-Demiralp et al., 2001). Furthermore, our *in vitro* results showing higher incidence of NETosis in BD could explain why BD patients have been previously reported as having high levels of extracellular NE in their circulation (Deger et al., 1995) and in their saliva as we have reported in this study. Activated neutrophils recruited to the mouth due to ongoing inflammation and/or microbial antigens, or neutrophils that passively gain access to the mouth via GCF or small cuts, may have a tendency to undergo NETosis rather than an alternative cell death. Once in the oral cavity, they contribute high levels of NE, histones, and ROS products in the surroundings of delicate oral tissues. Since it has been shown that NETs can directly kill epithelial and endothelial cells (Saffarzadeh et al., 2012), it is important to verify the contribution of NETosing neutrophils during oral ulceration.

5.4.2.2. NE Inhibitor Can Stop the Progression of NETosis *in Vitro*

Active NE is required to carry out NETosis (Papayannopoulos et al., 2010). A previous study showed that a synthetic NE-specific inhibitor blocked NE's ability to decondense chromatin which is one of the main occurrences needed for making NETs (Papayannopoulos et al., 2010). We employed the same inhibitor in our experiments as confirmation that not only was NE an integral part of both PMA-stimulated and unstimulated NETosis but that it could reduce the incidence of this cell death pathway.

The classic characteristic of early NET formation involves the intracellular translocation of NE from the azurophil granule to the nucleus (Fuchs et al., 2007). This was confirmed by the co-localisation of the DNA and NE antibody targets in our experiments. However, this observation was greatly reduced when NEi was used in cultures. The longer incubations with NEi (maximum 4 hrs) showed more frequent observations that the inhibitor was dampening NETosis. Another indication that the inhibitor was dampening neutrophil activity was the fact that very few cells were able to adhere to the glass coverslips. This was most evident in HC cultures. The most remarkable observable

difference between unstimulated cultures with or without NEi occurred in cells from BD patients. For example, one BD patient whose untreated neutrophils showed atypical morphology reminiscent of advanced NETosis exhibited rounded cells with hypersegmented nuclei after the addition of NEi. Collectively, the data confirms that unstimulated and PMA-induced NETosis in neutrophils from HC and BD patients involves endogenous NE which can be inhibited.

Artificially reducing the occurrence or progression of NETosis may help individuals with chronic inflammation or autoimmune diseases. In these cases, neutrophils and other immune cells congregate to rid its host of a foreign or host-derived antigen. An abundance of NETs can become destructive to host tissue, if not regulated, therefore, incorporating exogenous NE inhibitors as a form of treatment could help to dampen the NETosis pathway.

5.4.2.3. The Role of Neutrophil-Derived α 1AT and SLPI

5.4.2.3.1. Intracellular Participation of SLPI but not α 1AT

Alpha1-antitrypsin was not seen to co-localise with NE or DNA in intact neutrophils while in unstimulated or PMA-induced cultures. This suggests that α 1AT does not have active involvement during the NETosis process. However, in order to prove its lack of participation, a specific inhibitor for α 1AT would have to be employed. To our knowledge, this is the first time the locale of α 1AT has been described while NETosis is taking place. It was not until the NETs were released that the α 1AT could be detected localising with the NE/DNA similar to Frenzel's, *et al*, *in vitro* findings (Frenzel et al., 2012) suggesting α 1AT could be binding to and inhibiting NE when externalised.

NE and α 1AT have been described as having “one of the highest known physiological associations” by Janoff (Janoff, 1972). It has been shown that when α 1AT binds to NE the association cannot be reversed due to a change in α 1AT conformation rendering both proteins inactive (Carrell et al., 1982, Dickens and Lomas, 2011). If α 1AT were to bind to the NE intracellularly, NETosis would probably never take place due to their irreversible binding. NE's ability to break down the histones in the chromatin would be inhibited. Interestingly, endogenous α 1AT is stored in the same azurophil granule as NE (Mason et al., 1991). Since azurophil granules are not believed to fuse with the nuclear membrane during NETosis (Papayannopoulos et al., 2010), this further demonstrates the selective process of NE's translocation from the azurophil to the nucleus. Also, the glycosylated

α 1AT isoform in the azurophil granule is thought to be different from the 52 kDa isoform found in human plasma and the other neutrophil granules including the specific, gelatinase, and secretory vesicles (Clemmensen et al., 2011). Western blots of subcellular neutrophil fractions have revealed a doublet band of α 1AT at 37 and 44 kDa from azurophil granules (Clemmensen et al., 2011). This suggests that the two isoforms from different granules may behave differently.

While α 1AT was observed exclusively in the cytoplasm up until NETs were produced regardless of culture treatment, the intracellular co-localisation of SLPI with the NE/DNA complex occurred in unstimulated cultures as opposed to those with PMA. Unlike α 1AT, SLPI does not bind irreversibly to NE (Krowarsch et al., 2003), is stored in separate specific (secondary) granules (Jacobsen et al., 2008), and is a smaller protein than α 1AT to transport (12 kDa compared to 52 kDa). Its size and flexible interaction with NE could be the evolutionary reason SLPI accompanies it into the nucleus during NETosis as opposed to α 1AT.

5.4.2.3.2. Extracellular Co-Localisation of SLPI and α 1AT with NE

Although externalised NE in NETs was shown to be active (Saffarzadeh et al., 2012), it is not clear to what extent and whether they remain active when bound to DNA. The NE found as globular regions on NETs is enzymatically active or whether its anti-microbial property is due to the high cationic charge restraining microbes. Determining NE's activity is central to determining the potential damage it can cause to the surrounding host tissues. Recently, NE was reported to provide the majority of proteolytic activity on NETs when compared to PR3 and CatG (O'Donoghue et al., 2013). However, this information was based solely on three HC donors' neutrophils which were induced with PMA. Therefore, the enzymatic activity of externalised NE due to naturally-occurring NETosis still needs further exploration.

In our study, after externalisation of the neutrophil contents, SLPI and α 1AT can be seen to speckle-coat the NE and MPO as well as DNA fibres. However, the extent of each inhibitor's contribution to inactivate NE is not yet known. Sallenave, *et al*, reported neutrophil-derived SLPI as a major inhibitor of NE in a model of upper airway inflammation despite neutrophils also producing α 1AT and elafin (Sallenave et al., 1997). It has been theorised that when SLPI is bound to NE, the combined charge of the aggregated proteins is highly cationic (Sullivan et al., 2008). Bacteria with a negatively

charged cell surface could then be trapped to the SLPI/NE complex. Also, to note, negatively charged DNA could also bind to this complex (Belorgey and Bieth, 1995).

The fate of α 1AT in the company of MPO has been demonstrated in other studies. MPO produces hypochlorous acid which acts to inhibit α 1AT's ability to bind to elastase (Borregaard et al., 1987). Reactive oxygen species (ROS) (Vignola et al., 1998) as well as neutrophil-derived collagenase (Michaelis et al., 1990) and metalloproteinases (Vissers et al., 1988) can inactivate α 1AT. Likewise, plasma-derived α 1AT has also been shown to inhibit neutrophil superoxide production (Bucurenci et al., 1992). SLPI is also susceptible to inactivation by oxidants (Boudier and Bieth, 1994). Depending on the location, oxidase levels, and interactions, α 1AT and SLPI could be at risk of being inactivated extracellularly.

A study by Skrzeczynska-Moncznik, *et al*, showed differences in the location of SLPI staining on NETs and the type of stimulation (PMA versus psoriatic serum) (Skrzeczynska-Moncznik et al., 2012). Firstly, all *in vitro* resting neutrophils showed SLPI as a granular stain similar to our images. Secondly, in their cultures, PMA-stimulated NETs from HC and psoriasis patients showed only partial staining for extracellular SLPI. Meanwhile, HC neutrophils stimulated with psoriatic serum showed SLPI co-localise with NE and DNA in the majority of NETs. Their latter observation could have been due to their previous intracellular co-localisation of SLPI with NE/DNA during NETosis as was seen in our unstimulated culture experiments. Presumably, as neutrophils release NETs, SLPI remains attached to the NE/DNA complex. In our study, PMA-induced neutrophils did not incorporate SLPI into the nucleus alongside the DNA during NETosis, therefore perhaps it was also partially disassociated from the external NE and DNA as seen by Skrzeczynska-Moncznik, *et al*. SLPI was also seen to co-localise with extracellular NE and DNA in psoriatic skin biopsies.

A demonstration of the impact that extracellular SLPI/NE/DNA complexes have on the immune system was also explored in Skrzeczynska-Moncznik, *et al*'s, study (Skrzeczynska-Moncznik et al., 2012). The production of Type I IFN- α by blood-derived plasmacytoid dendritic cells was measured *in vitro* using a combination of rSLPI, isolated human NE, and DNA as stimulants. Type I IFN- α is known to induce autoimmunity in psoriasis but usually responds to viral DNA to inhibit pathogen replication. Alone or with various coupling, SLPI, NE, and/or DNA did not elicit a response, however the combined

use of all three triggered a significant increase of Type I IFN- α . Replacing SLPI with α 1AT failed to show an increase above baseline as did inactivating NE in the combined trio. This demonstrates that the SLPI/NE/DNA complex that results from NETs can induce a strong immune reaction. Since BD patients' neutrophils have shown evidence of the extracellular co-location of these elements, it would be worth exploring the SLPI/NE/DNA complex *in vitro* with BD epithelial and endothelial cells in order to measure various immunological cell responses and its potential contribution to BD pathology.

A multitude of NET-associated proteins have been identified by mass spectrometry such as CatG and β -actin (O'Donoghue et al., 2013, Saffarzadeh et al., 2012, Urban et al., 2009), however neither SLPI nor α 1AT have been detected. This may be due to the fact that the analysis has solely been on PMA-induced NETs from HC neutrophils. Other factors that may have affected the mass spectrometry analysis could have been the extensive preparation techniques including washing the NETs, digesting them with DNase or MNase and exposure to various reagents in preparation for peptide sequencing.

Some of the proteins identified in NETs are also targets for SLPI and α 1AT such as CatG and PR3. Therefore, the inhibitors could only partially be involved in regulating NE. Depending on the *in vivo* location, such as the circulation, tissue, or oral cavity, an influx of endogenous, non-neutrophil derived SLPI and α 1AT could also contribute to the protease inhibition. It is imperative to know if NE from NETs is being appropriately regulated in BD.

5.4.3. The Current Model of NETosis: NE, MPO, SLPI, and α 1AT

From our collective findings, NETosing neutrophils appear to undergoing a different pathway when in unstimulated cultures compared to PMA-induced NETosis. **Fig 5.4.3.1.** demonstrates our proposed unstimulated NETosis pathway *in vitro*.

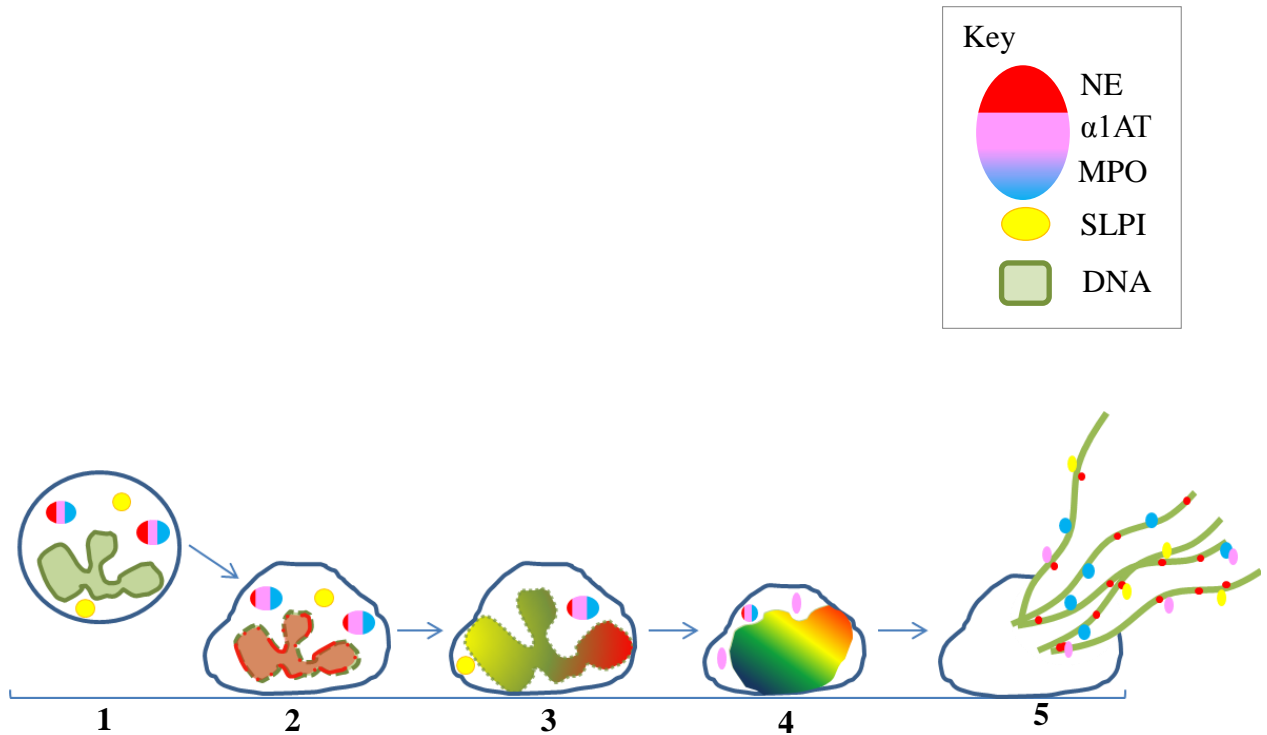


Figure 5.4.3.1. Suggested *in vitro* model of unstimulated, sterile NETosis observed in our experimental system. **1.** Active neutrophils are free floating in the culture media. They are usually round and have a multi-lobed nucleus (green). Some cells attach to surfaces. Azurophilic granules with NE (red), MPO (blue), and α 1AT (pink) are located in the cytoplasm along with specific granules containing SLPI (yellow). Note, that α 1AT may be found in all neutrophil granules (Clemmensen et al., 2011) but is only depicted in the azurophilic granule in this diagram. Granules within the cytoplasm outline the nucleus. **2.** When NETosis ensues, cells readily attach to non-coated glass surface. The periphery of the nucleus has the highest concentration of NE in early NETosis then diffuses throughout the DNA. Some NE granules remain in the cytoplasm (in PMA-stimulated cells, these granules appear more prominent). **3.** The supercoiled DNA unravels and the nucleus begins to lose its lobular structure. SLPI can be seen to co-localise with the NE in the nucleus (not seen in the PMA-induced pathway). α 1AT remains in the cytoplasm. At a late stage, MPO can be found co-localising with the NE and DNA. **4.** Mixed material co-localises and fills the cell. α 1AT still remains in the cytoplasm. **5.** NETs are expelled. Filaments of DNA with NE, MPO, SLPI, and α 1AT attached are muddled together in a web. (loosely based on a schematic by Brinkmann and Zychlinsky (Brinkmann and Zychlinsky, 2012) which has been updated to represent our observations).

5.4.4. Eosinophils Did Not Undergo NETosis

The co-precipitation of a low number of eosinophils during the blood neutrophil isolation was non-intentional. But despite a few eosinophils being observed in the cultures, they were never seen to undergo ETosis naturally or with PMA stimulation. Their appearance was easily identified having bi-lobed nucleus and staining positive for SLPI (and α 1AT) but not for NE. Eosinophils in patients' cultures the number of which depended on the individual were observed to have attached to the non-coated coverslips despite neutrophils having perished via NETosis or other cell death process or not having attached at all. As a secondary observation, it was interesting to see that the eosinophils present did not participate in ETosis. In a study by Yousefi, *et al.*, (Yousefi et al., 2008), they showed that human eosinophils primed by IL-5 and IFN- γ and stimulated by LPS, C5a, and eotaxin *in vitro* produced ETs. They also showed evidence of ETosis in Crohn's disease patient colon tissue samples. However, our observations would exclude eosinophil's participation in ETosis.

5.4.5. Unidentified Death

In unstimulated cultures, there were a few neutrophils which did not undergo NETosis but expired via an alternative pathway. Apoptosis, spontaneous death, autophagy, or secondary necrosis could have been the cause. Primary necrosis was unlikely as its occurrence usually involves a harsh environment such as high osmotic pressure or a toxic element such as endotoxin and often occurs in groups of cells. Autophagy was also unlikely to occur in the experiment's enriched culture conditions as this pathway is up regulated in nutrient deprivation. Apoptosis and secondary necrosis (Silva, 2010) were thought to be the two main alternative cell death pathways seen in our cultures. Typically in apoptosis the neutrophils undergo blebbing, as was seen in some cultures, where their DNA would degrade, condense, and the cell itself become small and round. If apoptosing neutrophils are not engulfed and removed by scavenger cells such as macrophages, then secondary necrosis may take place. This is commonly seen in isolated cultures (Silva, 2010). However, the occurrence of these processes are currently speculative since without additional investigations, such as using Annexin V, Propidium iodide, and CD16 markers, it is difficult to categorise all the terminal pathways taking place. However, the critical assessment was whether or not the destructive cellular enzymes, such as NE, were being externalised and to examine the attendance of endogenous regulators.

5.4.6. Conclusions

The evidence that BD patients' neutrophils readily undergo NETosis in unstimulated, sterile culture conditions implicates its occurrence *in vivo*. NETs are also thought to be a natural and beneficial part of the innate immune system since HC neutrophils have been seen to manifest NETs in a small portion of their neutrophil population. The lack of NET production can severely hinder the control of infections as is the case in CGD. However, an overabundance of NETs may lack purpose in a sterile environment. Unfortunately, the increase of extracellular components, especially NE, may be causing direct detrimental effects to the integrity of the host's vasculature and connective tissues. Therefore, NETs may be partially responsible for the chronic inflammation and pathology associated with BD, but it is imperative to deduce to what extent in order to know what treatment strategy to adapt. Also, gaining a better understanding of the roles that endogenous NE inhibitors play during both the NETosis pathway and in the extracellular environment is also important to define. Based on our investigations, it is believed that SLPI but not α 1AT coincides intracellularly with NE and DNA during non-PMA induced NETosis. The role these components play is still unclear. However, in the extracellular environment, the extent of involvement of SLPI, α 1AT, and other protease inhibitors will dictate the balance or imbalance of proteolytic activity.

Chapter 6: Validated Reference Genes for Ulcerated and Non- Ulcerated Oral Buccal Mucosa

6.1. Introduction

6.1.2. Reference Genes in RT-qPCR

Reference genes are also referred to as internal controls or housekeeping genes. They constitute conserved genes that are present in most, if not all, cells from various species at different expression levels depending on the cell or tissue type. In order to utilise these genes to help normalise the quantity and quality of messenger (m)RNA in a given cellular lysate, the reference genes must retain a relatively continuous expression across comparative cells, experimental treatments, or disease states. In other words, they should not be influenced and therefore fluctuate their expression levels due to these conditions. Validation of reference gene stability in samples being compared for their gene expression is paramount to represent a true and significant outcome for the gene of interest (GOI) after normalisation. The less stable the reference gene, the larger the fold-change needed in order to measure a significant difference (Bustin, 2004).

An internal control is also essential to correct for any error introduced during the entire experimental procedure starting with the collection of the specimens to be tested, for instance, whether they are frozen, lysed immediately, or paraffin-embedded. The specimen itself will have biological variations as well such as its transcriptional activity. There is a dependency or assumption that all chemical reactions will be identical, however enzymes such as endogenous RNase, exogenous DNase for degrading genomic DNA, reverse transcriptase, and polymerase will have slightly varied reactions depending on the starting material, batch differences, or external laboratory circumstances. Despite optimising the assay, primer efficiency can vary slightly as can Sybr green fluorescent dsDNA attachment. Error can also be introduced by the operator as perfect repetition is impossible. Other mechanical differences such as the retention of RNA in extraction columns, centrifuge speeds, the temperatures of heat blocks, or the running of the RT-PCR instruments are heavily relied upon for 100% performance but again are relying on all of the experimental reactions and preparations to be as similar as possible. Measuring a gene in the same RNA sample that has been subjected to all the same errors which can then be measured alongside the gene of interest is an important element to control for all these potential variations (Huggett et al., 2005).

Using more than one reference gene can be useful for normalisation especially if the reference genes have different biological cell functions. In this way, one can be sure that the genes are not co-regulated. Vandesompele, *et al*, recommends using three reference genes to improve the accuracy of expression levels (2002). However, there are practical considerations which may override this suggestion such as having a low amount of RNA per sample or if several of the reference genes show the same stability.

It is also advised that a reference gene should amplify around the same cycle quantification (Cq) as the GOI so as to avoid large variability. This can also provide a better representation of the gene integrity and site availability. Therefore, ribosomal (r)RNA is a debatable reference target since it is often highly abundant in comparison to mRNA (Overbergh *et al.*, 2003). To add to this, rRNA cannot be used when the RT step incorporates only oligo(dT) primers because rRNA lacks poly-A tails.

The 5'-3' assay which assesses the integrity of mRNA was developed by Nolan, *et al*, in 2006 (Nolan *et al.*, 2006a) and was modified and incorporated by Roche, UK on the human reference gene panel. Their modification incorporated a 5', middle, and 3' primer pairs for an unknown target. The principle of the assay was to measure the expression of a gDNA gene (a sequence which includes the intron sequence as opposed to just exon regions for mRNA) using these 3 different target sites on the same sequence. If the mRNA was intact and the sequence continual (during the RT reaction), then the 3 targets will result in similar amplification results. An acceptable difference between the 3 resulting Cq's is ≤ 1 as deemed by Roche's protocol.

On the panel, two reaction wells per sample were also included as RT negative (-) controls. This means that RNA, whether it has been DNase treated or not, is not subjected to an RT reaction (no cDNA is produced) thus there should be no amplification. Instead, a small aliquot is set aside for an amplification test to see if residual gDNA still exists in the sample. Again, the target of these reactions is Roche's proprietary information.

6.1.3. The History of Using RNA from Oral Buccal Epithelium for Molecular Investigations

Oral buccal epithelial cell RNA has been previously investigated but centres around studies associated with oral squamous cell carcinoma (OSCC). The majority of publications do not mention validation of their reference genes for their studies. One recent investigation by Rentoft, *et al*, in 2010 recognised this necessary verification by testing 8 potential reference genes in formalin-fixed paraffin-embedded oral biopsies (OSCC and normal tissue) from various sites which are known to have fragmented RNA (Rentoft et al., 2010). However, much more frequently, RT-qPCR studies have employed default reference genes such as GAPDH, HPRT1, 18S rRNA, and β -actin without verifying the expression of these particular genes with their unique experiments (Bar et al., 2009). Historically, these genes were used as controls for non- or semi-quantitative methods such as northern blot or early RT-PCR; however due to the highly sensitive nature of qPCR, they have been found to be irregularly expressed amongst the various experimental protocols and samples.

Two years after the initial publication of Vandesompele *et al*'s geNorm as a solution to years of qPCR experts' urgency for the need of proper reference gene validation and qPCR normalisation (Vandesompele et al., 2002), Spivack, *et al*, employed the use of GAPDH, β -actin, and 36B4 reference genes for the normalisation of carcinogen targets in OBS without any prior stability tests (Spivack et al., 2004). Also, instead of calculating a mean Cq of the three reference genes in order to normalise the GOI collectively, the ratio of their target was assessed separately to only one or two of the mentioned reference genes (Spivack et al., 2004). Many others have chosen GAPDH or β -actin as default internal controls without validation for qPCR experiments when measuring gene expression in OBS OSCC and inflammatory lesions (Driemel et al., 2007) or oral lichen planus biopsy tissue (Ding et al., 2010). The random selection and use of a literature-accepted reference gene was again demonstrated by Toyoshima, *et al*, in 2009 when OSCC patients with different cancer progressions had their buccal swabs assessed for cytokeratin expression alongside a head/neck SCC cell line and a normal oral mucosa cell line (Toyoshima et al., 2009a). With so many different sample types, the assessment of their randomly selected reference gene, GAPDH, should have been more carefully investigated before concluding their findings.

Around the same time, Campos, *et al*, finally recognised the fact that there was a lack of reference gene validation when using qPCR to analyse gene expression in OSCC clinical samples and cell lines (Campos et al., 2009). They set about to find the stable genes in immortalised oral cell lines, but more importantly addressed an important issue that many were by-passing. To date, there have been no reports of stable RNA reference genes investigated for OBS samples from HC, BD and RAS patients with and without oral ulcers.

6.1.4. Applying and Interpreting geNorm Analysis using qBasePLUS

GeNorm is an analytic algorithm developed by Jo Vandesompele, *et al*, in 2002 to determine the most stable gene amongst various samples in an experiment (Vandesompele, 2002, Genome Biology). GeNorm has been incorporated into the qBasePLUS software (Biogazelle, Belgium) for ease of use. The software applications, formulas, and analysis are described in detail in Hellemans, *et al*'s, paper in 2007 (Hellemans et al., 2007). In summary, after using qPCR to amplifying multiple candidate reference genes in the cDNA samples that are going to be used for a gene expression study, the duplicate or triplicate Cq results are uploaded into the qBasePLUS software. The geNorm program performs automatic computations to first transform the average of these Cq's into non-normalised values using the $2^{\Delta Cq}$ method where 2 is the assumed 100% assay efficiency raised to the power of delta (Δ) Cq. ΔCq is the difference between the lowest Cq and a Cq from the same gene in a different sample. This new value is called the relative quantity (RQ).

In order to determine which reference gene is most stable, the geNorm program uses an extensive algorithm which ranks the genes by applying a step by step elimination of the least stable gene (Vandesompele et al., 2002). The principle lies in the fact that if a ratio between two Cq values is calculated and the Cq values were the same, the ratio would be equal to 1 and there would be no variability. However, in a real scenario, there are differences in Cq results and an increase in their ratio dissimilarity decreases gene expression stability. Each candidate reference gene generates an average expression stability measure, M. The lower the M value, the more stable the gene expression. An acceptable M value for homogeneous samples is < 0.5 . A typical set up for these samples would be comparing the same cell culture for experimental treatment and control. For heterogeneous samples, such as human clinical specimens, an M value < 1.0 is generally accepted for the reference gene stability.

After establishing which reference genes are stable, qBasePlus can also normalise these reference gene Cq results to those of the target gene. The exact assay efficiency for each target can be input instead of assuming 100% allowing more accurate results. A normalization factor is generated for each sample by calculating the geometric mean of the RQ values for each stable reference gene measured in that one sample. The gene of interest RQ values can also be determined, but in order to obtain the normalised GOI results relative to the reference gene expression, the GOI RQ is divided by the geomean (normalisation factor) of the reference gene for each sample.

The reference genes remain valid for use unless the experimental conditions are altered, such as how the RNA samples are preserved or if a cell culture component is adjusted. If conditions are changed, then reference genes needs to be re-tested on geNorm to see if they are still stable.

6.1.5. Experiment Objectives

1. To determine appropriate, stable mRNA reference genes from ulcerated and non-ulcerated oral buccal mucosa swabs obtained from HCs as well as BD and RAS patients.
2. To determine whether the Roche Human Reference Gene Panel can provide suitable reference genes options out of the panel's 19 different genes being tested.
3. Test whether the in-built RNA Integrity Assay on the Roche Human Reference Gene Panel can be used to analyse the RNA quality of the OBS samples.

6.2. Materials and Methods

6.2.1. QPCR Roche Human Reference Gene Panel

The RealTime ready Human Reference Gene 384-well Panel (Roche, Germany) was chosen for the analysis of 19 different stable reference genes in cDNA from oral buccal mucosa swabs. Thirty-seven cDNA samples were analysed from three donor groups: HC, RAS, and BD. These were further categorised by the type of oral mucosa sampled, i.e., whether or not the swab was taken directly from an ulcer (U) or non-ulcerated area (NU). The samples run on the reference gene panel are listed in **Table 6.2.1.1.**

Patient Group and Oral Ulcer Activity	Area being swabbed	Sample number (n)
HC	NU	7
RASq	NU	6
RASa	NU	3
RASa	U	2
BDq	NU	10
BDa	NU	5
BDa	U	4

Table 6.2.1.1. Details of the OBS from Different Patient Groups, the Area Swabbed, and Sample Number (n) Tested on the Human Reference Gene Panel. HC (healthy control), RAS (recurrent aphthous stomatitis), BD (Behçet's disease). q, indicates mouth quiet/no ulcers. a, refers specifically to active mouth ulcer(s) regardless of other systemic symptoms. NU = swab taken from the buccal mucosa away from the oral ulcer. U = swab taken directly from the ulcer.

The protocol recommended by the manufacturer using between 50 pg to 50 ng total RNA per reaction well. Also, they suggest that a cDNA sample should not make up over 4% of the total reaction volume otherwise there is a risk of inhibiting the reaction.

For each cDNA sample, a mastermix was prepared in combination with the 2X LightCycler® 480 Probes Master reaction mix (**Table 6.2.1.2**). Extra volume was allowed to accommodate for loss from repetitive pipetting therefore for each well, 5 µl of a 1:10 dilution of cDNA was added along with 5 µl of the probes mastermix which resulted in a final dilution of 1:20 or 5% cDNA in each reaction well. This was mixed thoroughly by pipette and 10 µl of each cDNA mastermix was then added to each well in row A, column 1 – 23 (**Fig 6.2.1.3**). Column 24 (the RT negative (-) control) had 1 µl of previously set aside non-transcribed OBS RNA samples, 4 µl of water, and 5 µl of the 2X LightCycler® 480 Probes Master reaction mix.

Sample or Reagent	x 1 reaction well	x 25 reaction wells
1:10 diluted cDNA	5 µl	125 µl
2X LightCycler® 480 Probes Master reaction mix	5 µl	125 µl
Total Volume	10 µl	250 µl

Table 6.2.1.2. Preparing the cDNA Sample Mastermix for each OBS sample to be run on the Human Reference Gene Panel.

In order to avoid cross-contamination while adding the sample reaction mix to one row, the rows on either side were covered temporarily with plate seals. When complete, the plate was covered with LightCycler[®] 480 Sealing Foil and centrifuged for 2 min at 1,500 x g in order to pull any liquid down into the wells.

The panel was run on the LightCycler[®] 480 System I instrument (Roche) by uploading a pre-set, kit-specific macros program provided by Roche. The reaction volume was set to 10 µl and detection format was a monocolour hydrolysis probe on dynamic mode with the FAM 483-533 filter (acquisition in the green channel). The program involved a pre-incubation step at 95°C for 10 min followed by 45 cycles of denaturing at 95°C, 10s; primer annealing at 60°C, 30s; and extension at 72°C, 1s. The last 30 s of the programme cooled the reaction plate to 40°C. The data were analysed on qBasePlus software V2.1 (Biogazelle, Belgium).

SEE FIGURE 6.2.1.3.

Fig 6.2.1.3. The RealTime Ready Human Reference Gene 384-well plate. 10 µl of each cDNA OBS sample mastermix was added to each row from wells 1 – 22. Each column contained a different pre-absorbed specific primers for each reference gene target. Untranscribed RNA from the same OBS sample, previously set aside after the extraction, was added to wells 23-24. This consisted of 1 µl of the RNA, 4 µl of RNase free H₂O, and 5 µl of the 2X LightCycler[®] 480 Probes Master reaction mix.

6.3. Results

6.3.1. Most Stable Reference Genes in Oral Buccal Epithelial Cells

Three 384-well 19 reference genes panels were used to assess the most stable reference genes for 37 OBS cDNA samples from RAS and BD patients with and without oral ulcers alongside HC. Two samples, a HC and BDa,U, had several missing Cq values in the assay and therefore were excluded from the reference gene analysis leaving a total of 35 OBS to collectively assess. The amplified reference genes were excluded if their Cq value was > 40 (since this indicated that there was not enough sample to be amplified and/or the gene had a low stability).

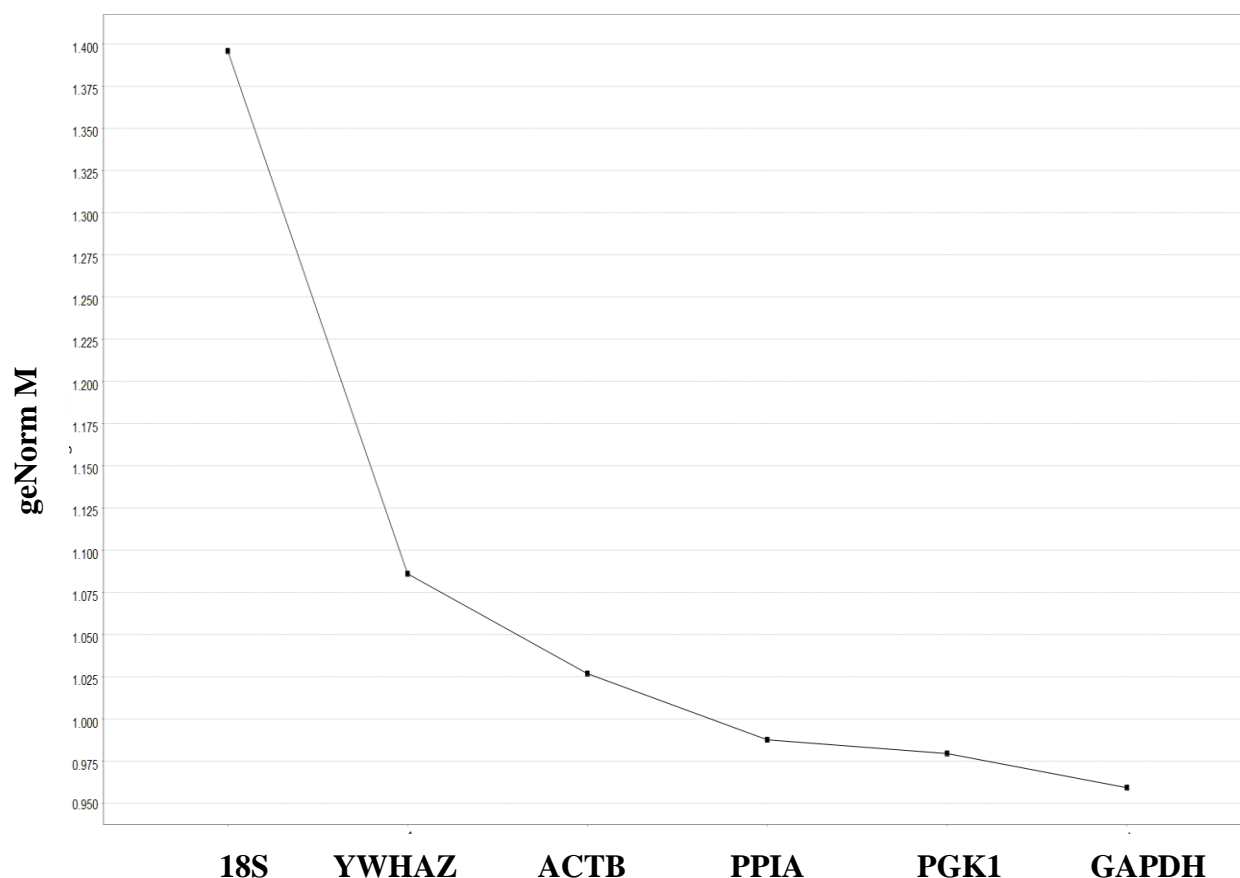


Fig 6.3.1.1. Average Expression Stability of Reference Gene Targets. The most stable gene across all samples has the lowest M value (GAPDH). The least stable of the top six reference genes tested was 18S rRNA.

To demonstrate the ranking of the candidate reference genes stability, the M value was calculated using qBasePLUS software. The M value was within the acceptable heterogeneous M value less than 1.0 for GAPDH, PGK1, and PPIA (**Fig 6.3.1.2**). The next three reference genes, β -actin, YWHAZ, and 18S rRNA, had a lower stability demonstrated by the M value being above 1.0. This is likely due to analysing clinical specimens in which the biological variability is much greater than cultured cell lines.

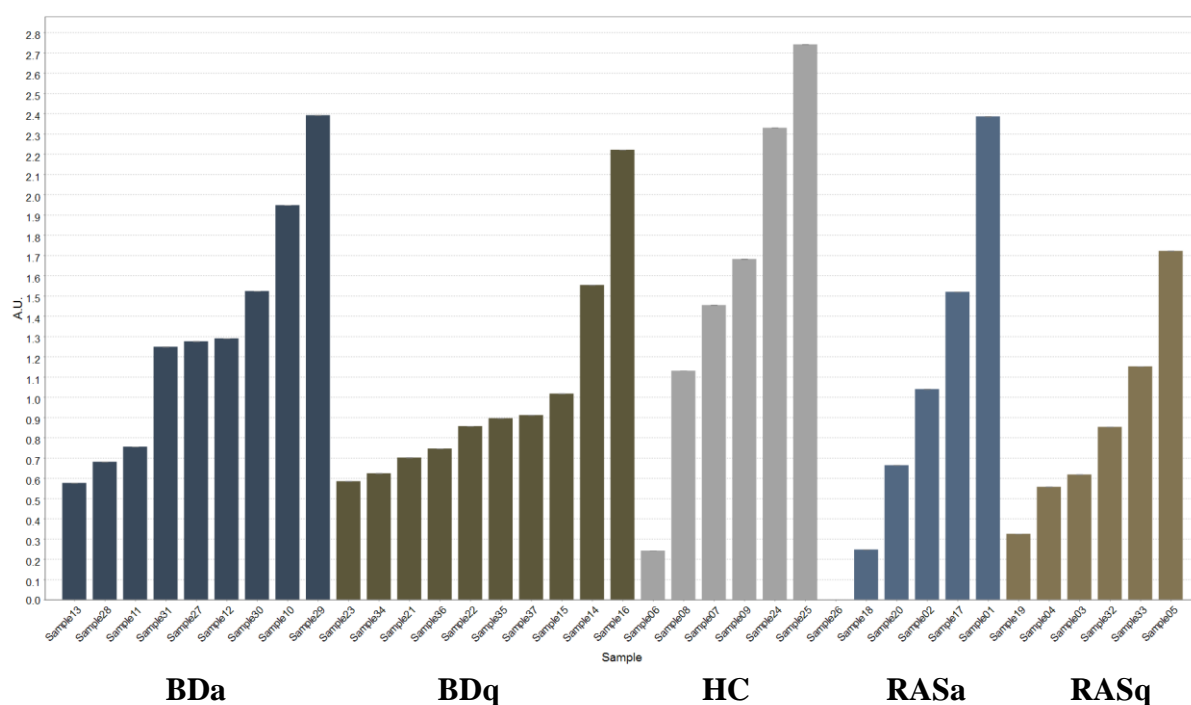


Fig 6.3.1.2. The Stability of GAPDH in BD and RAS patients with and without oral ulcers and HC as demonstrated on a qBasePLUS software bar graph. Y-axis constitutes arbitrary units (A.U.) The histogram shows a similar range (minimum and maximum) of GAPDH expression for each patient group.

The most stable of the reference genes for our OBS patient samples was GAPDH. This shows that despite the oral epithelium being ulcerated, the relative expression of this gene was unwavering. The next acceptable stable reference genes were PPIA and PGK1 and were also below the M recommended value of 1.0.

The least stable reference gene out of all 19 on the panel was hypoxanthine phosphoribosyltransferase 1 (HPRT1) which encodes the gene for the enzyme transferase (data not shown). It is important for creating purine nucleotides via the purine salvage pathway. Most of the samples failed to amplify the HPRT1 gene at all.

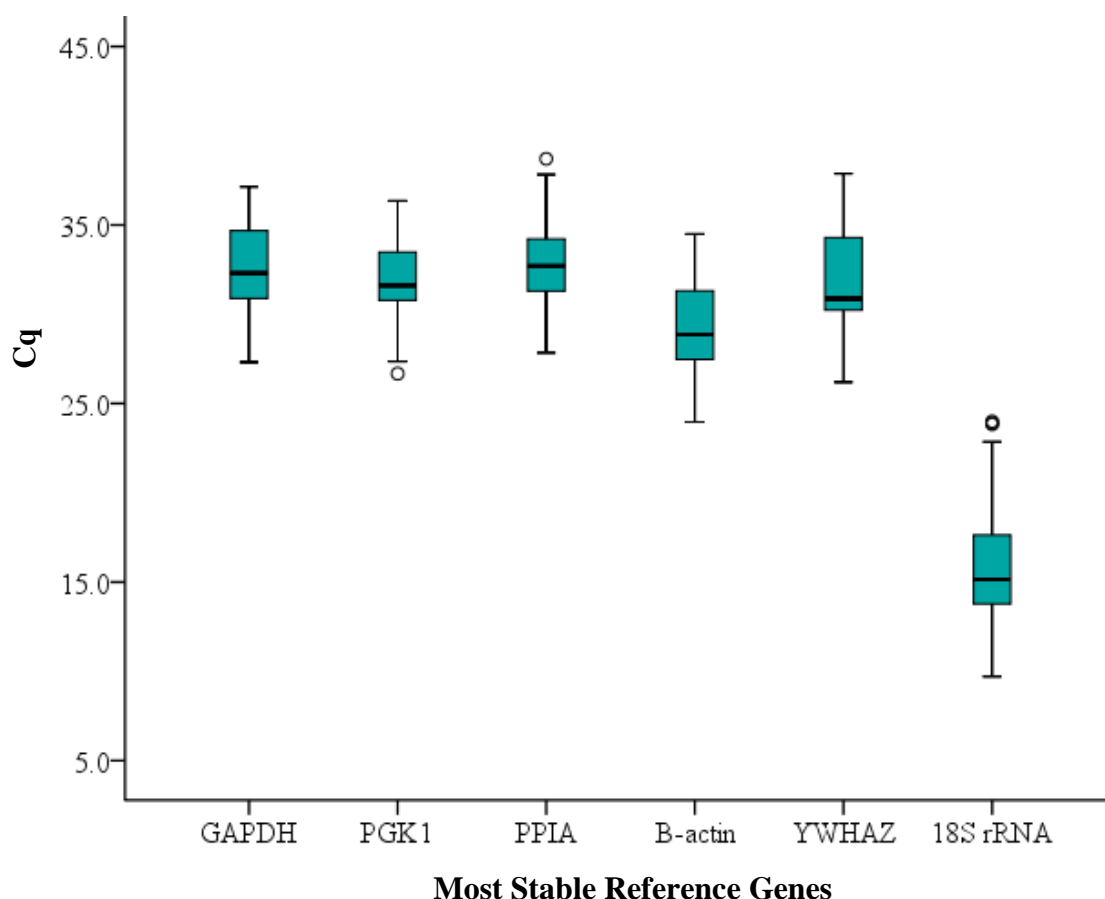


Fig. 6.3.1.3. Box Plot of Reference Gene Cq Results for all 7 Different Patient Group OBS Samples. The bar in the centre of each box indicates the median Cq (cycle quantification). The box itself represents 50% of the Cq values and the extended bars encompass maximum and minimum values. o indicates outliers.

Reference gene	Mean Cq	STDEV	SEM
GAPDH	32.6	2.58	0.44
PGK1	31.8	2.35	0.40
PPIA	33.0	2.71	0.46
β -Actin	29.4	2.88	0.49
YWHAZ	31.9	2.87	0.48
18S rRNA	16.1	3.91	0.66

Table 6.3.1.4. The Mean Cq, Standard Deviation, and Standard Error Mean of the Six Most Stable Reference Genes in OBS.

6.3.2. The RNA Integrity Assay

The results were varied when analysing the integrity assay which was incorporated into 3 separate reaction wells of the plate to amplify the 5', middle, 3' sections of an unknown gene in each cDNA sample. For example, one of the cDNA samples resulted in a Cq of

32.6 for the 5' end, 33.3 for the middle, and 33.6 for the 3' end (**Fig 6.3.2.1, A**). The calculated difference between the 5' end and the middle was 0.7 Cq's. For the middle and 3' end, the difference equated to 0.3 while the difference between the 5' and 3' was 1. According to Roche's definition, the similarity in $Cq \leq 1$ amongst the 3 different sites of the same gene suggests that this particular site of RNA that was transcribed during the RT process was intact and therefore can deduce that the RNA is of good quality. **Fig 6.3.2.1, B** shows another sample that amplified its 5', middle, and 3' end at 37.1, 36.2 and 40 Cq, respectively. The differences in Cq were 0.9, 3.8, and 2.9. This suggests that the RNA may have been slightly degraded before RT.

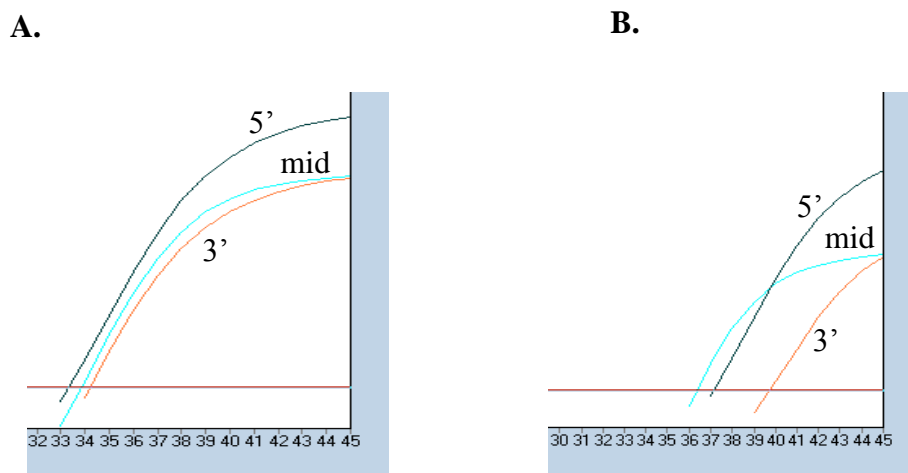


Fig 6.3.2.1. Demonstration of two different cDNA samples tested on the human reference gene panel for the continuous and equal reverse transcription of its RNA. A. One cDNA sample with similar amplification Cq's for all 3 sections (5', middle, 3' end) of one gene. **B.** A different cDNA sample showing the Cq's for the discontinuous sections of one gene.

Resulting Differences in Cq of the 5', middle, and 3' qPCR Reactions for Each cDNA Sample	Number of cDNA samples	Percentage of all samples
All 3 Cq's show a difference > 1	3 out of 35	8.6%
Only one difference amongst all 3 Cq's was ≤ 1	10 out of 35	28.6%
2 Cq's have a difference ≤ 1	4 out of 35	11.4%
All 3 Cq's have a difference ≤ 1	18 out of 35	51.4%

Table 6.3.2.1. The 5', middle, and 3' amplification results for all 35 cDNA samples included in the human reference gene panel.

When assessing all of the cDNA samples (**Table 6.3.2.1**), the majority of them (18/35 or 51.4%) had all 3 site amplification differences ≤ 1 Cq. At least 11.4% (4/35) of the samples had all three Cq results that were in close proximity during amplification but still had one result that was above a difference of 1. Just over a quarter of the samples, 28.6% (10/35), could only equate a difference ≤ 1 Cq in one out of the three Cq comparisons. Finally, only 3 out of 36 samples (8.6%) showed all 5', middle, and 3' Cq results had a difference greater than 1 when compared to each other.

None of the RT- RNA samples amplified and were therefore concluded to be clear of any gDNA contamination.

6.4. Discussion

The investment in three of these commercial reference gene panels was necessary to gain as much information as possible from our OBS samples which had low RNA to start with and also had never been tested on ulcerated mucosa cells in these patient groups. Ideally, 10 OBS samples from each group would have been used as recommend by Derveaux, *et al.*, (Derveaux et al., 2010) but this was hindered by three major issues: 1) obtaining consent from study participants for an OBS taken directly from an ulcer which was painful at times due to the pressure required to collect enough epithelial cells for the assay, 2) the high cost of the reference gene panels, and 3) no guarantee that the sample would not be excluded from the panel due to late Cq (> 40) or lack of gene amplification.

When an ulcer was swabbed, the RNA recovered was frequently either of poor quality and/or very low quantity due to not being able to apply the same pressure on the wound compared to an area of non-ulcerated tissue. The Micro RNeasy RNA extraction kit (Qiagen, UK) was used instead of the Illustra TriplePrep Kit (GE Healthcare, UK) in order to try to obtain concentrated RNA from each OBS. A small volume of 20 ng of carrier poly-A RNA (provided by the Qiagen kit) was added to each lysate prior to loading the sample on the column in order to improve the total RNA recovery. Despite these efforts, the average RNA quantity measured on the Nanodrop™ just after RNA extraction (prior to the DNase digest) was higher in the BDa non-ulcerated samples at 65.5 ng/μl compared to 38.1 ng/μl for those collected directly from ulcers. The RASa patients showed a similar result with non-ulcerated swabs having an average of 29.8 ng/μl and the ulcerated sample being only 11.3 ng/μl. After residual gDNA was eliminated, which incorporated another dilution of the RNA, the samples were checked on the Bioanalyzer and several direct ulcer samples had to be reconsidered for downstream analysis due to either extremely low quantity or dubious quality. Other studies experienced the same limitations of OBS RNA collection when attempting to gather cells from an oral wound (Spira et al., 2004).

OSCC samples, the basis for most oral RNA qPCR investigations, have been reported to have a high frequency of unusable samples. For instance, Toyoshima, *et al*, reported that 14 out of 66 (21.2%) exfoliated oral epithelial samples had to be excluded due to poor quality (Toyoshima et al., 2009b). Another study conducted by Driemel, *et al*, reported that out of 28 OSCC OBS patient samples, only 5 (17.9%) were suitable for use when assessed for RNA integrity. Also, they were only able to incorporate 10 out of 22 (45.5%) OBS from inflamed or hyperproliferative lesions. Meanwhile, 100% (5 out of 5) of HC OBS samples were acceptable (Driemel et al., 2007). Driemel, *et al*, attributed this loss of RNA integrity to a high volume of oral bacterial RNase in the samples, although this seems unlikely as RNase would indiscriminately destroy the RNA in both OSCC and HC samples alike. Nevertheless, these studies were still able to find significant differences in gene expression after eliminating poor quality OBS samples.

Despite the low quantity of RNA from ulcerated mucosa, six reference genes were still able to be found to have similarly stable expressions in the various samples. GAPDH, PGK1, and PPIA were regarded as the top three most stable. While β -actin, YWHAZ, and 18S rRNA could potentially be used as additional reference genes, the need to include them in normalisation assessment was not immediately necessary. However, seeing as how 18S rRNA was overly abundant in the OBS samples resulting in amplification between 9.7 and 24 Cq, using it for normalisation would not be appropriate unless a target gene was also found within the same range.

Similar to the diversity of our patient sample type (HC, BD, RAS, normal tissue or ulcerated site), Rentoft's, *et al*, study tested only a small number of oral biopsies for reference gene stability by geNorm in wax embedded oral tissue (Rentoft et al., 2010). The 10 OSCC and 10 control samples they analysed were further sub-divided by tissue type consisting of gingiva, tongue, hard palate, and buccal mucosa. Nonetheless, they were able to successfully categorise the best and worst reference genes for their samples. The most stable across all tissue types and malignancies was tubulin alpha-6 chain and the ribosomal protein S13 and the least being HPRT1 which was infrequently amplified (the latter being similar to our results).

There has been some controversy surrounding the use of GAPDH as a reference gene for normalisation. However, this has been due to the fact that either the gene has not been tested for its stable expression before use in normalisation, or following an investigation, has been deemed inappropriate for certain experiments using such samples as normal colon epithelium (Bustin, 2000). GAPDH has also shown an upregulation in human prostate cancer (Ripple and Wilding, 1995). We did not find that this was the case in our patient groups with intact or ulcerated oral buccal epithelium, therefore, we are confident that GAPDH can be used as an appropriate reference gene for RT-qPCR normalisation.

Similar in function to GAPDH, the protein encoded by the phosphoglycerate kinase 1 (PGK1) gene, the second most stable reference gene for our experiments, is an enzyme involved toward the end of the glycolysis pathway which catalyses the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. The third most stable gene was peptidylprolyl isomerase A, (PPIA) otherwise known as cyclophilin A, which encodes for proteins folding. The combined use of this gene plus either GAPDH or PGK1 could be useful in normalising future targets of interest in the oral buccal samples.

A recent reference gene stability profile conducted by Riemer, *et al*, investigated 2 normal and 6 HPV-immortalized keratinocyte cell lines with and without IFN- γ stimulus. Interestingly, almost identical to our findings, they found that GAPDH, PGK1, PPIA, and 18S were among the most stable of 16 different reference genes tested (Riemer et al., 2012). Naturally, their M values in geNorm assessment was much lower than ours seeing as they were working with established cell lines as opposed to a variety of clinical samples, however, is still an intriguing similarity due to the cell type and potential pro-inflammatory stimulus.

There was an issue addressed in the Spivack, *et al*, study in which they regarded the further dilution of precious RNA by applying a DNase reaction as unnecessary if primers are designed across exons (excluding introns) (Spivack et al., 2004). However, although this seems like a good suggestion, not knowing the design of the primer sequences in the ready-to-use commercial reference gene panel, we could not take the chance of inappropriate amplification of gDNA and therefore would always DNase-treat the RNA.

Also, seeing as we were using the same OBS samples in addition to new samples for measuring our GOI in subsequent assays, we needed to keep the treatment of the RNA sample the same in order to lower the risk of variability.

The 5'-middle-3' assay (well reactions 20, 21, 22) aimed to amplify 3 different sections of the same gene in our samples (the gene on the panel was undisclosed due to proprietary information). Ideally, the use of Oligo(dT) primers during the reverse transcription (RT) step is important for the assay's interpretation because it would show the continuous polyA-tail 3' end of the mRNA transcribed without interruption towards the 5' end. If the physical integrity of the RNA was completely intact, then the assay would result in 3 similar Cq's for all 3 targets of the same gene. However, because we solely employed the use of random primers in the RT step (anticipating some degree of RNA degradation), the resulting Cq of the 3 targets on the same gene showed greater variability due to the fact that the random primers could have started the transcription at indiscriminate and different areas. Therefore, our results from this assay on the panel was helpful but could not provide a definitive estimate of our OBS RNA quality and integrity. For this, we relied on pre-RT RNA Bioanalyzer data. In future experiments, it would be advised for others to use a mix of Oligo(dT) and random primers prior to using the Roche reference gene panels if they wanted to use this feature as an additional assessment of RNA integrity. On a different quality assessment, the RT- test on non-RT treated RNA was negative for any amplification. Therefore, no residual gDNA existed in any of the samples.

6.4.1. Conclusions

Oral buccal epithelial cells collected using a cytological brush, can provide a decent quality and quantity of RNA from which the mRNA expression of reference genes can be reliably assessed. A large number of OBS samples should be collected from areas of ulcerated tissue to improve the number of samples with enough RNA that can successfully be included in multiple qPCR assays. By checking the stability of a wide range of 19 different reference genes in OBS samples from BD and RAS patients with and without oral ulcers as well as HC, we are able to apply the recommended qPCR normalisation technique comparing the most stable reference genes with our target genes and report significant findings with confidence.

Chapter 7:

Final

Discussion

7.1. Final Discussion

Neutrophils are an important and vital cellular component of the innate immune system providing rapid protection against microbial invasion. As phagocytes and antigen presenting cells, they are recruited to the sites of injury or inflammation by IL-8, TNF- α , C5a, or bacterial LPS and work with a multitude of other host cells and additional signal proteins to fight infection and then regain a state of system equilibrium. Their contribution to the complex network of the immune system's up regulation, sustaining, and subsequent down regulation is a normal occurrence in healthy individuals.

Maintaining this balance is essential, as neutrophils contain potent proteolytic enzymes and the potential to produce ROS which can, if exposed to the local environment, cause cellular destruction indiscriminate of pathogen or host. When released extracellularly or if it is surface-bound to neutrophils, the azurophil granule component, NE, contributes to neutrophil chemotaxis. However, NE also has an affinity for ECM as a substrate with the ability to cleave elastin, collagen, fibronectin, and laminin all of which compose the host's connective tissue (Roghani and Sallanave, 2008b, Heutinck et al., 2010). Therefore, an overabundance of NE can breakdown the tissue.

Endogenous specific inhibitors of NE, such as SLPI and α 1AT, are present within human plasma as well as neutrophils themselves (Clemmensen et al., 2011). These protease inhibitors interact with exposed NE by physically attaching to it in a 1:1 stoichiometric configuration. By doing so, they immobilise and, at least partially, inactivate NE (Morrison et al., 1990) depending on the local environmental factors such as pH and presence of oxidants which are known to enable SLPI and α 1AT. This protease/antiprotease complex is then cleared by phagocytic macrophages. If there is excessive NE, the inhibitors may struggle to control it incidentally allowing damage to take place.

Individuals with BD have been shown to have hyperchemotaxis of neutrophils (Matsumura and Mizushima, 1975) (Neves et al., 2009), have an increased expression of HLA-B51 antigen which has been linked to neutrophil over-activity (O'Duffy et al., 1983) (Sensi et al., 1991), and specifically, the increase of circulating NE during disease relapse and remission (Deger et al., 1995). We also identified for the first time that BD patients have a high incidence of NETs *in vitro* which may contribute to the externalisation and increase of NE *in vivo*. Since oral ulcers are a key manifestation in BD, it is thought that if the reoccurrence of ulceration can be controlled, then the other symptoms may dampen

as well. The concentration of NE into the mouth during quiescent episodes had not previously been investigated. If excessive NE is shed into the oral cavity and its protease inhibitors are unable to maintain a balanced NE concentration and activity, the integrity of the oral mucosal epithelium may be compromised leading to an ulcer.

The research elucidated from this study has provided evidence of an increased presence of NE in saliva from persons who suffer from BD. In addition, these high levels of NE were still detected in BD patients on medication to dampen their immune system. The reoccurrence of BD oral ulcers indeed may be due to an overabundance of NE and its proteolytic activity despite the fact that BDq patients' saliva simultaneously carries at least seven times the concentration of the endogenous inhibitor α 1AT which is thought to permanently inactivate NE when bound (Korkmaz, Moreau et al. 2008). Furthermore, SLPI, locally released from the saliva glands and mucosa epithelial cells, appears to be reduced in BD patients without oral ulcers. In fact, whenever NE was high, SLPI was low across all patient groups tested, including RAS. To add to the matter, we found that oral epithelial cells from non-ulcerated mucosa in BD and RAS patients had significantly upregulated expression of SLPI mRNA. This suggests that either the production of SLPI cannot meet the demand or that salivary SLPI protein is quickly depleted by the increased presence of NE in these patients.

The single SLPI protein band on our western blots supports the theory that SLPI is only being detected when it is unbound. If SLPI and NE are complexed, it was not apparent using detection antibodies in either NE or SLPI western blots. In saliva, NE:SLPI complexed configuration may still convey protection as an anti-microbial agent due to the dual cationic charge that can disrupt bacterial membranes. The complex should also decrease the enzymatic activity of NE. However, according to shared theories by Gauthier, *et al*, and Krowarsch, *et al*, , SLPI may have reversible inhibition with NE where they can detach from each other without conformational or functional alterations (Krowarsch et al., 2003) (Gauthier et al., 1982). Although NE is an important component of the innate immune system to kill bacteria and resolve infection (Belaaouaj, 2002), it is a concern that neither α 1AT nor SLPI are able to regulate active NE when its concentration increases in BD patient saliva without the accompaniment of infection or ulceration. This may be due to the deactivation of α 1AT and SLPI by oxidants present in saliva (Carp and Janoff, 1980) (Matheson et al., 1979).

The presence of intracellular SLPI was detected in oral buccal epithelial cells adjacent to a BD ulcer. This helps to support our finding that SLPI mRNA is expressed in these cells and SLPI protein is being produced. It was also confirmed that minor buccal saliva gland was producing SLPI during a BD oral ulcer. Thus in these patients, SLPI does not seem to be deficient or depleted until it reaches the saliva which even in BDq patients is inundated with NE. Although NE infiltration was frequently seen in the submucosa of non-relapsed BD with oral ulcers, it was not seen above the intact basal cell barrier. However, our biopsy interpretations are based on ulcers that had manifested several days prior to the excision. The distribution of NE within the mucosa during quiescent BD could be more informative by revealing the immune cells and any extracellular proteases that are present which may be contributing to epithelial inflammation and erosion.

There are several possibilities why SLPI protein levels are depleted in BD saliva. The first possibility is that BD patients are deficient in SLPI which is made more evident during oral ulceration. Having ruled out oral epithelial cells' SLPI mRNA dysfunction, our data would suggest that there may still be post-translational modifications following the synthesis of SLPI such as abnormal protein folding or decreased accessory proteins to aid the intracellular production and transport. Exocytosis of the SLPI protein may be naturally slow, or during incidences where increased NE is present, may be reduced since both BD and RAS patients with ulcers had decreased salivary SLPI.

Another possibility is that SLPI may be complexed with excessive oral NE as well as other proteases such as CatG. SLPI may also be binding to oral commensal bacteria depleting its usefulness as an anti-protease. Upon binding, this may hide the SLPI antibody epitope. This would be the best scenario since potentially a different antibody can be produced with an alternative site. However, the SLPI antibody we used successfully detected SLPI co-localising with NE in neutrophils during NETosis and in extracellular NETs. Of course, co-localising does not necessarily mean they are bound to each other but it is a possibility. If antibody binding is not an issue and SLPI is genuinely deficient in BD, then patients may benefit from topical rSLPI as a replacement therapy. This could remedy the cycle of inflammation and reduce reoccurring ulceration.

It is important to investigate the role that neutrophils and their proteases have in producing the symptoms of BD, but it is equally important to explore the site of chemotactic activity, such as the oral cavity, where a discrepancy has been identified

between the high concentration of inflammatory cytokines and chemokines in saliva compared to serum. Gaining a better understanding the role of NE and its inhibitors in BD and RAS saliva can help elucidate the mechanism of oral ulceration and its involvement in systemic disease.

7.2. Final Conclusions

From our research, we conclude that the constant presence and excessive NE activity in the oral cavity is contributing to mucosal epithelium proteolytic damage thereby encouraging the development of oral ulcers. The depletion of its protease inhibitor, SLPI, and possible inactivation of a second specific inhibitor α 1AT in saliva may trump the dysregulation of NE. This study is the first to report the levels of NE and its inhibitors, SLPI and α 1AT, in BD and RAS saliva. BD patients' taking colchicine to suppress neutrophil chemotaxis did not lower the high levels of NE or pro-inflammatory cytokines detected their saliva and therefore may benefit from using an oral, topical NE suppressant. Saliva is a useful, non-invasive specimen in which to monitor NE levels and inflammatory cytokines.

This study also established appropriate reference genes for assessment of SLPI mRNA in oral buccal epithelial cells from BD and RAS patients compared to HCs. To date, no reference genes had been validated for use from BD and RAS patients' buccal mucosa with and without ulcers. Completing the reference gene panel facilitated an accurate assessment of BD patients' increased SLPI mRNA expression during period of remission. Oral buccal swabs are a useful, non-invasive technique that can potentially be used for molecular investigations in a variety of disorders with oral manifestations.

This is also the first study to detect NETs in BD which may be contributing to their systemic pathology. Also, during the production of NETs, our novel observation that intracellular SLPI but not α 1AT co-localised with NE suggests an intracellular regulatory role.

7.3. Future Work

With the recent update of international guidelines for diagnosing BD (Davatchi et al., 2013) and contributions from clinical and laboratory BD research with improved technology, revisiting the immunological aspects of BD is imperative as improved treatment strategies are needed. Identifying NETs in BD has provided evidence supporting the theory of neutrophil-derived pathology. Further investigations on NETs in BD are vital to assess the extent of NETosis occurring *in vivo* and if oral neutrophils are also releasing NETs thus exposing the oral environment to NE. The first critical and straightforward experiment to perform would be to expose neutrophils from HC to BD patient serum to see if there are serum components triggering NETs. It would also be helpful to know to what extent NE is active when released from NETs or bound to SLPI. Since current long-term BD medications can be detrimental, a better comprehension of the NETosis mechanism in BD can open the door for developing therapeutic interventions.

In order to further explore the imbalance of NE inhibitors in saliva, it would be advantageous to isolate and purify native SLPI from the saliva samples instead of using recombinant SLPI to assess inhibition of NE. The same should be assessed with endogenous α 1AT to test its inhibitory activity in saliva and reveal if it is functionally active or oxidised. Also, it would be informative to look at the inflammatory markers from RAS and BD patient biopsies during a quiescent episode to further investigate how the ulcers develop.

Appendix 1

Appendix 2

Appendix 2.1. Antibody Staining Controls

Isotype controls, primary-only and secondary-only antibodies were tested on both neutrophils and oral biopsies for any non-specific fluorescence. The optimised blocking and staining protocols successfully eliminated any non-specific antibody binding. The autofluorescence of tissues and cells was also tested and found to be negative. Two biopsies that underwent isotype control staining and primary antibody-only tests are demonstrated below in **Fig. Appendix 2.1**.

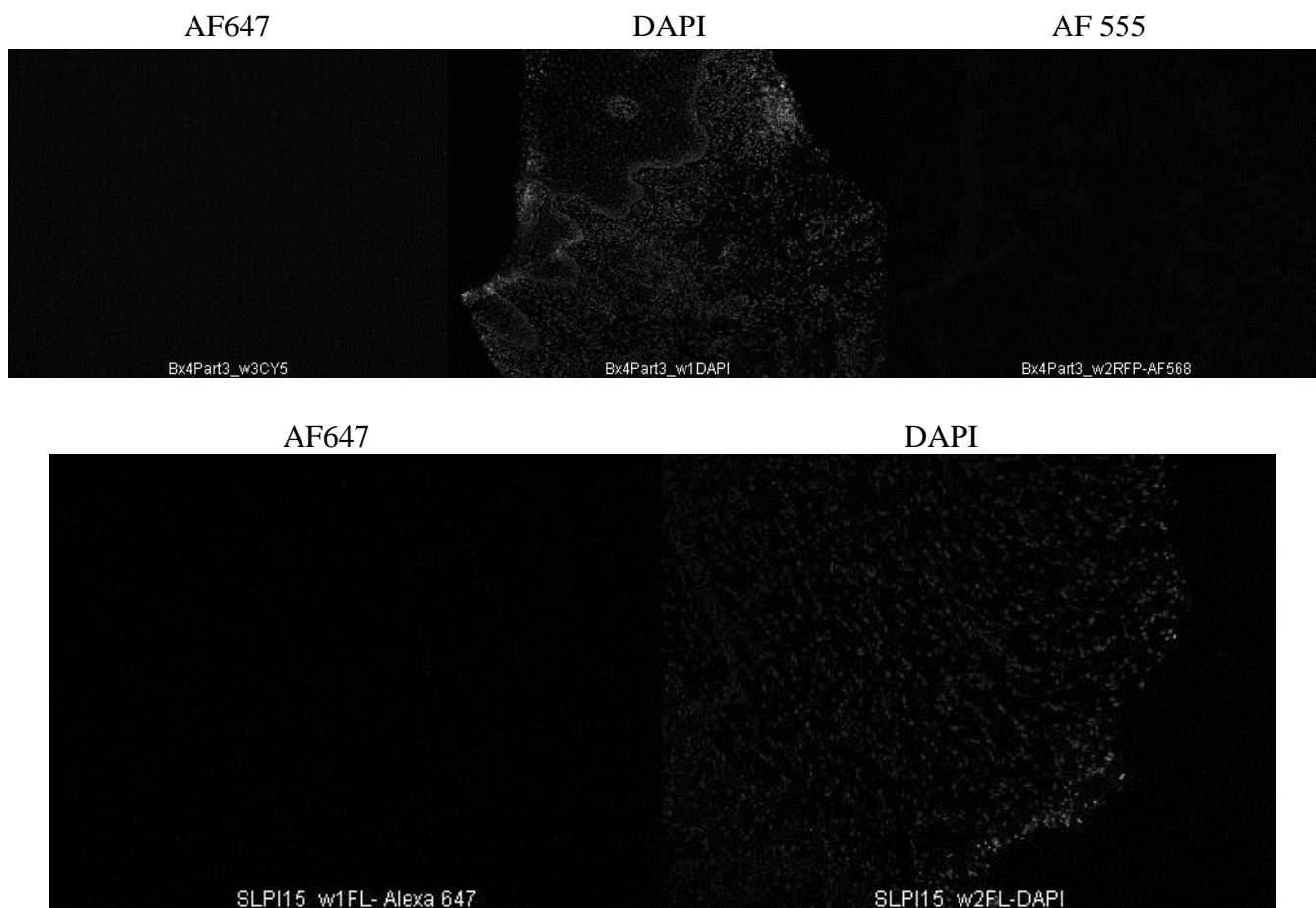


Fig. Appendix 2.1. Negative Control Tests. A. Secondary-Only Antibodies and B. Isotype Controls. Panel A shows a biopsy section with no primary antibody (blocking buffer was used instead) with the two secondary antibodies used in conjunction with SLPI and NE antibodies: polyclonal AF555 goat, anti-mouse IgG and polyclonal AF647 goat, anti-rabbit IgG, respectively. After various exposures and gain adjustments, neither antibody was detected. DAPI DNA dye is the only dye that could be seen. Panel B demonstrates the Mouse IgG1 (at the same concentration that was used for SLPI primary antibody) coupled with the optimised concentration of AF647 secondary antibody. Again, DAPI was identified but no non-specific binding was detected in the Cy5 far red channel.

References

1990. Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease. *Lancet*, 335, 1078-80.
- ACCORINTI, M., PIRRAGLIA, M. P., PAROLI, M. P., PRIORI, R., CONTI, F. & PIVETTI-PEZZI, P. 2007. Infliximab treatment for ocular and extraocular manifestations of Behcet's disease. *Japanese journal of ophthalmology*, 51, 191-6.
- ADISEN, E., ARAL, A., AYBAY, C. & GURER, M. A. 2008. Salivary epidermal growth factor levels in Behcet's disease and recurrent aphthous stomatitis. *Dermatology*, 217, 235-40.
- ADLER, Y. D., MANSMANN, U. & ZOUBOULIS, C. C. 2001. Mycophenolate mofetil is ineffective in the treatment of mucocutaneous Adamantiades-Behcet's disease. *Dermatology*, 203, 322-4.
- AKDENIZ, N., ESREFOGLU, M., KELES, M. S., KARAKUZU, A. & ATASOY, M. 2004. Serum interleukin-2, interleukin-6, tumour necrosis factor-alpha and nitric oxide levels in patients with Behcet's disease. *Annals of the Academy of Medicine, Singapore*, 33, 596-9.
- AKMAN, A., KACAROGLU, H., DONMEZ, L., BACANLI, A. & ALPSOY, E. 2007. Relationship between periodontal findings and Behcet's disease: a controlled study. *Journal of clinical periodontology*, 34, 485-91.
- AL-OTAIBI, L. M., PORTER, S. R. & POATE, T. W. 2005. Behcet's disease: a review. *J Dent Res*, 84, 209-22.
- ALBERTS, B. 1994. *Molecular biology of the cell*, New York ; [Great Britain], Garland Pub.
- ALLEN, D. H. & TRACY, P. B. 1995. Human coagulation factor V is activated to the functional cofactor by elastase and cathepsin G expressed at the monocyte surface. *J Biol Chem*, 270, 1408-15.
- ALPSOY, E., KODELJA, V., GOERDT, S., ORFANOS, C. E. & ZOUBOULIS CH, C. 2003. Serum of patients with Behcet's disease induces classical (pro-inflammatory) activation of human macrophages in vitro. *Dermatology*, 206, 225-32.
- ALTENBURG, A., ABDEL-NASER, M. B., SEEGER, H., ABDALLAH, M. & ZOUBOULIS, C. C. 2007. Practical aspects of management of recurrent aphthous stomatitis. *Journal of the European Academy of Dermatology and Venereology : JEADV*, 21, 1019-26.
- ALUNNO, A., BISTONI, O., BARTOLONI, E., CATERBI, S., BIGERNA, B., TABARRINI, A., MANNUCCI, R., FALINI, B. & GERLI, R. 2013. IL-17-producing CD4-CD8- T cells are expanded in the peripheral blood, infiltrate salivary glands and are resistant to corticosteroids in patients with primary Sjogren's syndrome. *Annals of the rheumatic diseases*, 72, 286-92.
- AMBROSI, A., ESPINOSA, A. & WAHREN-HERLENIUS, M. 2012. IL-17: a new actor in IFN-driven systemic autoimmune diseases. *European journal of immunology*, 42, 2274-84.
- AMULIC, B., CAZALET, C., HAYES, G. L., METZLER, K. D. & ZYCHLINSKY, A. 2012. Neutrophil function: from mechanisms to disease. *Annual review of immunology*, 30, 459-89.
- ANGELOV, N., MOUTSOPOULOS, N., JEONG, M. J., NARES, S., ASHCROFT, G. & WAHL, S. M. 2004. Aberrant mucosal wound repair in the absence of secretory leukocyte protease inhibitor. *Thromb Haemost*, 92, 288-97.
- ARABACI, T., KARA, C. & CICEK, Y. 2009. Relationship between periodontal parameters and Behcet's disease and evaluation of different treatments for oral recurrent aphthous stomatitis. *Journal of periodontal research*, 44, 718-25.

- ARIDOGAN, B. C., YILDIRIM, M., BAYSAL, V., INALÖZ, H. S., BAZ, K. & KAYA, S. 2003. Serum Levels of IL-4, IL-10, IL-12, IL-13 and IFN-gamma in Behcet's disease. *The Journal of dermatology*, 30, 602-7.
- ARIEL, A., YAVIN, E. J., HERSHKOVIZ, R., AVRON, A., FRANITZA, S., HARDAN, I., CAHALON, L., FRIDKIN, M. & LIDER, O. 1998. IL-2 induces T cell adherence to extracellular matrix: inhibition of adherence and migration by IL-2 peptides generated by leukocyte elastase. *Journal of immunology*, 161, 2465-72.
- ASHCROFT, G. S., LEI, K., JIN, W., LONGENECKER, G., KULKARNI, A. B., GREENWELL-WILD, T., HALE-DONZE, H., MCGRADY, G., SONG, X. Y. & WAHL, S. M. 2000. Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. *Nat Med*, 6, 1147-53.
- ATTUCCI, S., GAUTHIER, A., KORKMAZ, B., DELEPINE, P., MARTINO, M. F., SAUDUBRAY, F., DIOT, P. & GAUTHIER, F. 2006. EPI-hNE4, a proteolysis-resistant inhibitor of human neutrophil elastase and potential anti-inflammatory drug for treating cystic fibrosis. *J Pharmacol Exp Ther*, 318, 803-9.
- AVCI, O., ELLIDOKUZ, E., SIMSEK, I., BUYUKGEBIZ, B. & GUNES, A. T. 1999. Helicobacter pylori and Behcet's disease. *Dermatology*, 199, 140-3.
- BAGGIOLINI, M., WALZ, A. & KUNKEL, S. L. 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *The Journal of clinical investigation*, 84, 1045-9.
- BALAN, U., GONSALVES, N., JOSE, M. & GIRISH, K. L. 2012. Symptomatic changes of oral mucosa during normal hormonal turnover in healthy young menstruating women. *The journal of contemporary dental practice*, 13, 178-81.
- BANK, U., KUPPER, B., REINHOLD, D., HOFFMANN, T. & ANSORGE, S. 1999. Evidence for a crucial role of neutrophil-derived serine proteases in the inactivation of interleukin-6 at sites of inflammation. *FEBS letters*, 461, 235-40.
- BAR, M., BAR, D. & LEHMANN, B. 2009. Selection and validation of candidate housekeeping genes for studies of human keratinocytes--review and recommendations. *J Invest Dermatol*, 129, 535-7.
- BARRETT, A. W., CRUCHLEY, A. T. & WILLIAMS, D. M. 1996. Oral mucosal Langerhans' cells. *Crit Rev Oral Biol Med*, 7, 36-58.
- BELAAOUAJ, A. 2002. Neutrophil elastase-mediated killing of bacteria: lessons from targeted mutagenesis. *Microbes and infection / Institut Pasteur*, 4, 1259-64.
- BELKOWSKI, S. M., BOOT, J. D., MASCELLI, M. A., DIAMANT, Z., DE GARAVILLA, L., HERTZOG, B., POLKOVITCH, D., TOWERS, M., BATHEJA, A. & D'ANDREA, M. R. 2009. Cleaved secretory leukocyte protease inhibitor as a biomarker of chymase activity in allergic airway disease. *Clin Exp Allergy*, 39, 1179-86.
- BELORGEY, D. & BIETH, J. G. 1995. DNA binds neutrophil elastase and mucus proteinase inhibitor and impairs their functional activity. *FEBS letters*, 361, 265-8.
- BENEDETTI, G. & MIOSSEC, P. 2013. Interleukin 17 contributes to the chronicity of inflammatory diseases such as rheumatoid arthritis. *European journal of immunology*.
- BERGENFELDT, M., NYSTROM, M., BOHE, M., LINDSTROM, C., POLLING, A. & OHLSSON, K. 1996. Localization of immunoreactive secretory leukocyte protease inhibitor (SLPI) in intestinal mucosa. *Journal of gastroenterology*, 31, 18-23.
- BIANCHI, M., HAKKIM, A., BRINKMANN, V., SILER, U., SEGER, R. A., ZYCHLINSKY, A. & REICHENBACH, J. 2009. Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood*, 114, 2619-22.
- BINGLE, L. & TETLEY, T. D. 1996. Secretory leukoprotease inhibitor: partnering alpha 1-proteinase inhibitor to combat pulmonary inflammation. *Thorax*, 51, 1273-4.

- BORREGAARD, N., JENSEN, H. S. & BJERRUM, O. W. 1987. Prevention of tissue damage: inhibition of myeloperoxidase mediated inactivation of alpha 1-proteinase inhibitor by N-acetyl cysteine, glutathione, and methionine. *Agents and actions*, 22, 255-60.
- BOUDIER, C. & BIETH, J. G. 1994. Oxidized mucus proteinase inhibitor: a fairly potent neutrophil elastase inhibitor. *The Biochemical journal*, 303 (Pt 1), 61-8.
- BOULINGUEZ, S., REIX, S., BEDANE, C., DEBROCK, C., BOUYSSOU-GAUTHIER, M. L., SPARSA, A., LE BRUN, V., DE VENCAY, P., BERNARD, P. & BONNETBLANC, J. M. 2000. Role of drug exposure in aphthous ulcers: a case-control study. *The British journal of dermatology*, 143, 1261-5.
- BOUMA, G., ANCLIFF, P. J., THRASHER, A. J. & BURNS, S. O. 2010. Recent advances in the understanding of genetic defects of neutrophil number and function. *Br J Haematol*, 151, 312-26.
- BOXER, L. & DALE, D. C. 2002. Neutropenia: causes and consequences. *Seminars in hematology*, 39, 75-81.
- BRANZK, N. & PAPAYANNOPOULOS, V. 2013. Molecular mechanisms regulating NETosis in infection and disease. *Seminars in immunopathology*, 35, 513-30.
- BRINKMANN, V., GOOSMANN, C., KUHN, L. I. & ZYCHLINSKY, A. 2012. Automatic quantification of in vitro NET formation. *Frontiers in immunology*, 3, 413.
- BRINKMANN, V., REICHARD, U., GOOSMANN, C., FAULER, B., UHLEMANN, Y., WEISS, D. S., WEINRAUCH, Y. & ZYCHLINSKY, A. 2004. Neutrophil extracellular traps kill bacteria. *Science*, 303, 1532-5.
- BRINKMANN, V. & ZYCHLINSKY, A. 2012. Neutrophil extracellular traps: is immunity the second function of chromatin? *The Journal of cell biology*, 198, 773-83.
- BUCURENCI, N., BLAKE, D. R., CHIDWICK, K. & WINYARD, P. G. 1992. Inhibition of neutrophil superoxide production by human plasma alpha 1-antitrypsin. *FEBS letters*, 300, 21-4.
- BUNO, I. J., HUFF, J. C., WESTON, W. L., COOK, D. T. & BRICE, S. L. 1998. Elevated levels of interferon gamma, tumor necrosis factor alpha, interleukins 2, 4, and 5, but not interleukin 10, are present in recurrent aphthous stomatitis. *Archives of dermatology*, 134, 827-31.
- BUSTIN, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of molecular endocrinology*, 25, 169-93.
- BUSTIN, S. A. 2004. *A-Z of quantitative PCR*, La Jolla, CA, International University Line.
- CAMPBELL, E. J., SILVERMAN, E. K. & CAMPBELL, M. A. 1989. Elastase and cathepsin G of human monocytes. Quantification of cellular content, release in response to stimuli, and heterogeneity in elastase-mediated proteolytic activity. *J Immunol*, 143, 2961-8.
- CAMPOS, M. S., RODINI, C. O., PINTO-JUNIOR, D. S. & NUNES, F. D. 2009. GAPD and tubulin are suitable internal controls for qPCR analysis of oral squamous cell carcinoma cell lines. *Oral oncology*, 45, 121-6.
- CANETE, J. D., CELIS, R., NOORDENBOS, T., MOLL, C., GOMEZ-PUERTA, J. A., PIZCUETA, P., PALACIN, A., TAK, P. P., SANMARTI, R. & BAETEN, D. 2009. Distinct synovial immunopathology in Behcet disease and psoriatic arthritis. *Arthritis research & therapy*, 11, R17.
- CARP, H. & JANOFF, A. 1980. Inactivation of bronchial mucous proteinase inhibitor by cigarette smoke and phagocyte-derived oxidants. *Exp Lung Res*, 1, 225-37.

- CARRELL, R. W., JEPSSON, J. O., LAURELL, C. B., BRENNAN, S. O., OWEN, M. C., VAUGHAN, L. & BOSWELL, D. R. 1982. Structure and variation of human alpha 1-antitrypsin. *Nature*, 298, 329-34.
- CAUCI, S., GUASCHINO, S., DE ALOYSIO, D., DRIUSSI, S., DE SANTO, D., PENACCHIONI, P. & QUADRIFOGLIO, F. 2003. Interrelationships of interleukin-8 with interleukin-1beta and neutrophils in vaginal fluid of healthy and bacterial vaginosis positive women. *Molecular human reproduction*, 9, 53-8.
- CAVARRA, E., LUCATTELLI, M., GAMBELLI, F., BARTALESI, B., FINESCHI, S., SZARKA, A., GIANNERINI, F., MARTORANA, P. A. & LUNGARELLA, G. 2001. Human SLPI inactivation after cigarette smoke exposure in a new in vivo model of pulmonary oxidative stress. *American journal of physiology. Lung cellular and molecular physiology*, 281, L412-7.
- CHAN, S. C., SHUM, D. K. & IP, M. S. 2003. Sputum sol neutrophil elastase activity in bronchiectasis: differential modulation by syndecan-1. *Am J Respir Crit Care Med*, 168, 192-8.
- CHAPMAN, H. A., JR., MUNGER, J. S. & SHI, G. P. 1994. The role of thiol proteases in tissue injury and remodeling. *American journal of respiratory and critical care medicine*, 150, S155-9.
- CHI, W., ZHU, X., YANG, P., LIU, X., LIN, X., ZHOU, H., HUANG, X. & KIJLSTRA, A. 2008. Upregulated IL-23 and IL-17 in Behcet patients with active uveitis. *Investigative ophthalmology & visual science*, 49, 3058-64.
- CHOI, P. & REISER, H. 1998. IL-4: role in disease and regulation of production. *Clinical and experimental immunology*, 113, 317-9.
- CICHY, J., POTEMPA, J. & TRAVIS, J. 1997. Biosynthesis of alpha1-proteinase inhibitor by human lung-derived epithelial cells. *The Journal of biological chemistry*, 272, 8250-5.
- CLEMMENSEN, S. N., JACOBSEN, L. C., RORVIG, S., ASKAA, B., CHRISTENSON, K., IVERSEN, M., JORGENSEN, M. H., LARSEN, M. T., VAN DEURS, B., OSTERGAARD, O., HEEGAARD, N. H., COWLAND, J. B. & BORREGAARD, N. 2011. Alpha-1-antitrypsin is produced by human neutrophil granulocytes and their precursors and liberated during granule exocytosis. *European journal of haematology*, 86, 517-30.
- COPE, A. P., LONDEI, M., CHU, N. R., COHEN, S. B., ELLIOTT, M. J., BRENNAN, F. M., MAINI, R. N. & FELDMANN, M. 1994. Chronic exposure to tumor necrosis factor (TNF) in vitro impairs the activation of T cells through the T cell receptor/CD3 complex; reversal in vivo by anti-TNF antibodies in patients with rheumatoid arthritis. *The Journal of clinical investigation*, 94, 749-60.
- COX, S. W., RODRIGUEZ-GONZALEZ, E. M., BOOTH, V. & ELEY, B. M. 2006. Secretory leukocyte protease inhibitor and its potential interactions with elastase and cathepsin B in gingival crevicular fluid and saliva from patients with chronic periodontitis. *J Periodontal Res*, 41, 477-85.
- CRONSTEIN, B. N., MOLAD, Y., REIBMAN, J., BALAKHANE, E., LEVIN, R. I. & WEISSMANN, G. 1995. Colchicine alters the quantitative and qualitative display of selectins on endothelial cells and neutrophils. *The Journal of clinical investigation*, 96, 994-1002.
- CURNOW, S. J., PRYCE, K., MODI, N., KNIGHT, B., GRAHAM, E. M., STEWART, J. E., FORTUNE, F., STANFORD, M. R., MURRAY, P. I. & WALLACE, G. R. 2008. Serum cytokine profiles in Behcet's disease: is there a role for IL-15 in pathogenesis? *Immunology letters*, 121, 7-12.
- DAGALIS, P., BAGG, J. & WALKER, D. M. 1987. Spontaneous migration and chemotactic activity of neutrophil polymorphonuclear leukocytes in recurrent

- aphthous ulceration. *Oral surgery, oral medicine, and oral pathology*, 64, 298-301.
- DALGHOUS, A. M., FREYSDOTTIR, J. & FORTUNE, F. 2006. Expression of cytokines, chemokines, and chemokine receptors in oral ulcers of patients with Behcet's disease (BD) and recurrent aphthous stomatitis is Th1-associated, although Th2-association is also observed in patients with BD. *Scandinavian journal of rheumatology*, 35, 472-5.
- DAMIANO, V. V., KUCICH, U., MURER, E., LAUDENSLAGER, N. & WEINBAUM, G. 1988. Ultrastructural quantitation of peroxidase- and elastase-containing granules in human neutrophils. *The American journal of pathology*, 131, 235-45.
- DAVATCHI, F., ASSAAD-KHALIL, S., CALAMIA, K. T., CROOK, J. E., SADEGHI-ABDOLLAHI, B., SCHIRMER, M., TZELLOS, T., ZOUBOULIS, C. C., AKHLAGI, M., AL-DALAAN, A., ALEKBEROVA, Z. S., ALI, A. A., ALTENBURG, A., ARROMDEE, E., BALTACI, M., BASTOS, M., BENAMOUR, S., BEN GHORBEL, I., BOYVAT, A., CARVALHO, L., CHEN, W., BEN-CHETRIT, E., CHAMS-DAVATCHI, C., CORREIA, J. A., CRESPO, J., DIAS, C., DONG, Y., PAIXAO-DUARTE, F., ELMUNTASER, K., ELONAKOV, A. V., GRANA GIL, J., HAGHDOOST, A. A., HAYANI, R. M., HOUMAN, H., ISAYEVA, A. R., JAMSHIDI, A. R., KAKLAMANIS, P., KUMAR, A., KYRGIDIS, A., MADANAT, W., NADJI, A., NAMBA, K., OHNO, S., OLIVIERI, I., VAZ PATTO, J., PIPITONE, N., DE QUEIROZ, M. V., RAMOS, F., RESENDE, C., ROSA, C. M., SALVARANI, C., SERRA, M. J., SHAHRAM, F., SHAMS, H., SHARQUIE, K. E., SLITI-KHANFIR, M., TRIBOLET DE ABREU, T., VASCONCELOS, C., VEDES, J., WECHSLER, B., CHENG, Y. K., ZHANG, Z. & ZIAEI, N. 2013. The International Criteria for Behcet's Disease (ICBD): a collaborative study of 27 countries on the sensitivity and specificity of the new criteria. *Journal of the European Academy of Dermatology and Venereology : JEADV*.
- DE WATER, R., WILLEMS, L. N., VAN MUIJEN, G. N., FRANKEN, C., FRANSEN, J. A., DIJKMAN, J. H. & KRAMPS, J. A. 1986. Ultrastructural localization of bronchial antileukoprotease in central and peripheral human airways by a gold-labeling technique using monoclonal antibodies. *Am Rev Respir Dis*, 133, 882-90.
- DECHATELET, L. R., SHIRLEY, P. S. & JOHNSTON, R. B., JR. 1976. Effect of phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leukocytes. *Blood*, 47, 545-54.
- DEGER, O., OREM, A., AKYOL, N., BAHADIR, S. & YILDIRMIS, S. 1995. Polymorphonuclear leukocyte elastase levels in patients with Behcet's disease. *Clin Chim Acta*, 236, 129-34.
- DEKKER, L. V., LEITGES, M., ALTSCHULER, G., MISTRY, N., MCDERMOTT, A., ROES, J. & SEGAL, A. W. 2000. Protein kinase C-beta contributes to NADPH oxidase activation in neutrophils. *The Biochemical journal*, 347 Pt 1, 285-9.
- DERVEAUX, S., VANDESOMPELE, J. & HELLEMANS, J. 2010. How to do successful gene expression analysis using real-time PCR. *Methods*, 50, 227-30.
- DICKENS, J. A. & LOMAS, D. A. 2011. Why has it been so difficult to prove the efficacy of alpha-1-antitrypsin replacement therapy? Insights from the study of disease pathogenesis. *Drug Des Devel Ther*, 5, 391-405.
- DICKINSON, D. P. 2002. Cysteine peptidases of mammals: their biological roles and potential effects in the oral cavity and other tissues in health and disease. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists*, 13, 238-75.
- DINARELLO, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood*, 87, 2095-147.

- DING, M., XU, J. Y. & FAN, Y. 2010. Altered expression of mRNA for HIF-1 α and its target genes RTP801 and VEGF in patients with oral lichen planus. *Oral diseases*, 16, 299-304.
- DIRESKENELI, H. 2006. Autoimmunity vs autoinflammation in Behcet's disease: do we oversimplify a complex disorder? *Rheumatology*, 45, 1461-5.
- DOUMAS, S., KOLOKOTRONIS, A. & STEFANOPOULOS, P. 2005. Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor. *Infect Immun*, 73, 1271-4.
- DOVI, J. V., SZPADERSKA, A. M. & DIPIETRO, L. A. 2004. Neutrophil function in the healing wound: adding insult to injury? *Thrombosis and haemostasis*, 92, 275-80.
- DRIEMEL, O., KOSMEHL, H., ROSENHAHN, J., BERNDT, A., REICHERT, T. E., ZARDI, L. & DAHSE, R. 2007. Expression analysis of extracellular matrix components in brush biopsies of oral lesions. *Anticancer research*, 27, 1565-70.
- DU BOIS, R. M., BERNAUDIN, J. F., PAAKKO, P., HUBBARD, R., TAKAHASHI, H., FERRANS, V. & CRYSTAL, R. G. 1991. Human neutrophils express the alpha 1-antitrypsin gene and produce alpha 1-antitrypsin. *Blood*, 77, 2724-30.
- DURMAZLAR, S. P., ULKAR, G. B., ESKIOGLU, F., TATLICAN, S., MERT, A. & AKGUL, A. 2009. Significance of serum interleukin-8 levels in patients with Behcet's disease: high levels may indicate vascular involvement. *International journal of dermatology*, 48, 259-64.
- DUZGUN, N., AYASLIOGLU, E., TUTKAK, H. & AYDINTUG, O. T. 2005. Cytokine inhibitors: soluble tumor necrosis factor receptor 1 and interleukin-1 receptor antagonist in Behcet's disease. *Rheumatology international*, 25, 1-5.
- EDRES, M. A., SCULLY, C. & GELBIER, M. 1997. Use of proprietary agents to relieve recurrent aphthous stomatitis. *British dental journal*, 182, 144-6.
- EKSIOGLU-DEMIRALP, E., DIRESKENELI, H., KIBAROGLU, A., YAVUZ, S., ERGUN, T. & AKOGLU, T. 2001. Neutrophil activation in Behcet's disease. *Clin Exp Rheumatol*, 19, S19-24.
- ELBORN, J. S., PERRETT, J., FORSMAN-SEMB, K., MARKS-KONCZALIK, J., GUNAWARDENA, K. & ENTWISTLE, N. 2012. Efficacy, safety and effect on biomarkers of AZD9668 in cystic fibrosis. *Eur Respir J*, 40, 969-76.
- ELENKOV, I. J. 2002. Systemic stress-induced Th2 shift and its clinical implications. *International review of neurobiology*, 52, 163-86.
- ERGUN, T., GURBUZ, O., HARVELL, J., JORIZZO, J. & WHITE, W. 1998. The histopathology of pathergy: a chronologic study of skin hyperreactivity in Behcet's disease. *International journal of dermatology*, 37, 929-33.
- FATAHZADEH, M., SCHWARTZ, R. A., KAPILA, R. & ROCHFORD, C. 2009. Orofacial Crohn's disease: an oral enigma. *Acta dermatovenerologica Croatica : ADC*, 17, 289-300.
- FAURSCHOU, M. & BORREGAARD, N. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect*, 5, 1317-27.
- FAURSCHOU, M., SORENSEN, O. E., JOHNSEN, A. H., ASKAA, J. & BORREGAARD, N. 2002. Defensin-rich granules of human neutrophils: characterization of secretory properties. *Biochim Biophys Acta*, 1591, 29-35.
- FELDMANN, M. & MAINI, R. N. 2001. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annual review of immunology*, 19, 163-96.
- FORMANEK, M., KNERER, B. & KORNFEHL, J. 1999. Cytokine expression of human oral keratinocytes. *ORL; journal for oto-rhino-laryngology and its related specialties*, 61, 103-7.

- FRASSANITO, M. A., DAMMACCO, R., CAFFORIO, P. & DAMMACCO, F. 1999. Th1 polarization of the immune response in Behcet's disease: a putative pathogenetic role of interleukin-12. *Arthritis and rheumatism*, 42, 1967-74.
- FRENZEL, E., KORENBAUM, E., HEGERMANN, J., OCHS, M., KOEPKE, J., KOCZULLA, A. R., WELTE, T., KOHNLEIN, T. & JANCIAUSKIENE, S. 2012. Does augmentation with alpha1-antitrypsin affect neutrophil extracellular traps formation? *International journal of biological sciences*, 8, 1023-5.
- FRESKO, I., SOY, M., HAMURYUDAN, V., YURDAKUL, S., YAVUZ, S., TUMER, Z. & YAZICI, H. 1998. Genetic anticipation in Behcet's syndrome. *Annals of the rheumatic diseases*, 57, 45-8.
- FUCHS, T. A., ABED, U., GOOSMANN, C., HURWITZ, R., SCHULZE, I., WAHN, V., WEINRAUCH, Y., BRINKMANN, V. & ZYCHLINSKY, A. 2007. Novel cell death program leads to neutrophil extracellular traps. *The Journal of cell biology*, 176, 231-41.
- FUCHS, T. A., BRILL, A., DUERSCHMIED, D., SCHATZBERG, D., MONESTIER, M., MYERS, D. D., JR., WROBLESKI, S. K., WAKEFIELD, T. W., HARTWIG, J. H. & WAGNER, D. D. 2010. Extracellular DNA traps promote thrombosis. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 15880-5.
- GADEK, J. E., FELLIS, G. A., ZIMMERMAN, R. L., RENNARD, S. I. & CRYSTAL, R. G. 1981. Antielastases of the human alveolar structures. Implications for the protease-antiprotease theory of emphysema. *J Clin Invest*, 68, 889-98.
- GARCIA-ROMO, G. S., CAIELLI, S., VEGA, B., CONNOLLY, J., ALLANTAZ, F., XU, Z., PUNARO, M., BAISCH, J., GUIDUCCI, C., COFFMAN, R. L., BARRAT, F. J., BANCHEREAU, J. & PASCUAL, V. 2011. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Science translational medicine*, 3, 73ra20.
- GAUTHIER, F., FRYKSMARK, U., OHLSSON, K. & BIETH, J. G. 1982. Kinetics of the inhibition of leukocyte elastase by the bronchial inhibitor. *Biochim Biophys Acta*, 700, 178-83.
- GAVRIN, L. K., DENNY, R. A. & SAIAH, E. 2012a. Small molecules that target protein misfolding. *Journal of medicinal chemistry*, 55, 10823-43.
- GAVRIN, L. K., DENNY, R. A. & SAIAH, E. 2012b. Small molecules that target protein misfolding. *J Med Chem*, 55, 10823-43.
- GHASEMLOU, N., BOUHY, D., YANG, J., LOPEZ-VALES, R., HABER, M., THURASINGAM, T., HE, G., RADZIOCH, D., DING, A. & DAVID, S. 2010. Beneficial effects of secretory leukocyte protease inhibitor after spinal cord injury. *Brain*, 133, 126-38.
- GILLISSEN, A., BIRRER, P., MCELVANEY, N. G., BUHL, R., VOGELMEIER, C., HOYT, R. F., JR., HUBBARD, R. C. & CRYSTAL, R. G. 1993. Recombinant secretory leukoprotease inhibitor augments glutathione levels in lung epithelial lining fluid. *Journal of applied physiology*, 75, 825-32.
- GINZBERG, H. H., CHERAPANOV, V., DONG, Q., CANTIN, A., MCCULLOCH, C. A., SHANNON, P. T. & DOWNEY, G. P. 2001. Neutrophil-mediated epithelial injury during transmigration: role of elastase. *American journal of physiology. Gastrointestinal and liver physiology*, 281, G705-17.
- GINZBERG, H. H., SHANNON, P. T., SUZUKI, T., HONG, O., VACHON, E., MORAES, T., ABREU, M. T., CHERAPANOV, V., WANG, X., CHOW, C. W. & DOWNEY, G. P. 2004. Leukocyte elastase induces epithelial apoptosis: role of mitochondrial permeability changes and Akt. *Am J Physiol Gastrointest Liver Physiol*, 287, G286-98.

- GOMEZ, S. A., ARGUELLES, C. L., GUERRIERI, D., TATEOSIAN, N. L., AMIANO, N. O., SLIMOVICH, R., MAFFIA, P. C., ABBATE, E., MUSELLA, R. M., GARCIA, V. E. & CHULUYAN, H. E. 2009. Secretory leukocyte protease inhibitor: a secreted pattern recognition receptor for mycobacteria. *American journal of respiratory and critical care medicine*, 179, 247-53.
- GOOPTU, B., HAZES, B., CHANG, W. S., DAFFORN, T. R., CARRELL, R. W., READ, R. J. & LOMAS, D. A. 2000. Inactive conformation of the serpin alpha(1)-antichymotrypsin indicates two-stage insertion of the reactive loop: implications for inhibitory function and conformational disease. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 67-72.
- GRAY, R. D., LUCAS, C. D., MACKELLAR, A., LI, F., HIERSEMENZEL, K., HASLETT, C., DAVIDSON, D. J. & ROSSI, A. G. 2013. Activation of conventional protein kinase C (PKC) is critical in the generation of human neutrophil extracellular traps. *Journal of inflammation*, 10, 12.
- GREENE, C. M. & MCELVANEY, N. G. 2010. Protein misfolding and obstructive lung disease. *Proceedings of the American Thoracic Society*, 7, 346-55.
- GRIESE, M., LATZIN, P., KAPPLER, M., WECKERLE, K., HEINZLMAIER, T., BERNHARDT, T. & HARTL, D. 2007. alpha1-Antitrypsin inhalation reduces airway inflammation in cystic fibrosis patients. *Eur Respir J*, 29, 240-50.
- GROBMYER, S. R., BARIE, P. S., NATHAN, C. F., FUORTES, M., LIN, E., LOWRY, S. F., WRIGHT, C. D., WEYANT, M. J., HYDO, L., REEVES, F., SHILOH, M. U. & DING, A. 2000a. Secretory leukocyte protease inhibitor, an inhibitor of neutrophil activation, is elevated in serum in human sepsis and experimental endotoxemia. *Critical care medicine*, 28, 1276-82.
- GROBMYER, S. R., BARIE, P. S., NATHAN, C. F., FUORTES, M., LIN, E., LOWRY, S. F., WRIGHT, C. D., WEYANT, M. J., HYDO, L., REEVES, F., SHILOH, M. U. & DING, A. 2000b. Secretory leukocyte protease inhibitor, an inhibitor of neutrophil activation, is elevated in serum in human sepsis and experimental endotoxemia. *Crit Care Med*, 28, 1276-82.
- GRON, B., STOLTZE, K., ANDERSSON, A. & DABELSTEEN, E. 2002. Oral fibroblasts produce more HGF and KGF than skin fibroblasts in response to co-culture with keratinocytes. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica*, 110, 892-8.
- GRUTTER, M. G., FENDRICH, G., HUBER, R. & BODE, W. 1988. The 2.5 Å X-ray crystal structure of the acid-stable proteinase inhibitor from human mucous secretions analysed in its complex with bovine alpha-chymotrypsin. *EMBO J*, 7, 345-51.
- GUPTA, A. K., HASLER, P., HOLZGREVE, W., GEBHARDT, S. & HAHN, S. 2005. Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Human immunology*, 66, 1146-54.
- HAJJAR, E., BROEMSTRUP, T., KANTARI, C., WITKO-SARSAT, V. & REUTER, N. 2010. Structures of human proteinase 3 and neutrophil elastase--so similar yet so different. *FEBS J*, 277, 2238-54.
- HAKKIM, A., FURNROHR, B. G., AMANN, K., LAUBE, B., ABED, U. A., BRINKMANN, V., HERRMANN, M., VOLL, R. E. & ZYCHLINSKY, A. 2010. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 9813-8.
- HAMURYUDAN, V., MAT, C., SAIP, S., OZYAZGAN, Y., SIVA, A., YURDAKUL, S., ZWINGENBERGER, K. & YAZICI, H. 1998. Thalidomide in the treatment of the mucocutaneous lesions of the Behcet syndrome. A randomized, double-blind, placebo-controlled trial. *Annals of internal medicine*, 128, 443-50.

- HASLETT, C. 1997. Granulocyte apoptosis and inflammatory disease. *Br Med Bull*, 53, 669-83.
- HATEMI, G., SILMAN, A., BANG, D., BODAGHI, B., CHAMBERLAIN, A. M., GUL, A., HOUMAN, M. H., KOTTER, I., OLIVIERI, I., SALVARANI, C., SFIKAKIS, P. P., SIVA, A., STANFORD, M. R., STUBIGER, N., YURDAKUL, S. & YAZICI, H. 2009. Management of Behcet disease: a systematic literature review for the European League Against Rheumatism evidence-based recommendations for the management of Behcet disease. *Annals of the rheumatic diseases*, 68, 1528-34.
- HATEMI, G. & YAZICI, H. 2011. Behcet's syndrome and micro-organisms. *Best practice & research. Clinical rheumatology*, 25, 389-406.
- HEINZEL-WIELAND, R., STEFFENS, G. J. & FLOHE, L. 1991. Inhibitory characteristics and oxidant resistance of site specific variants of recombinant human antileukoproteinase (ALP). *Biomedica biochimica acta*, 50, 677-81.
- HELLEMANS, J., MORTIER, G., DE PAEPE, A., SPELEMAN, F. & VANDESOMPELE, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome biology*, 8, R19.
- HEUTINCK, K. M., TEN BERGE, I. J., HACK, C. E., HAMANN, J. & ROWSHANI, A. T. 2010. Serine proteases of the human immune system in health and disease. *Mol Immunol*, 47, 1943-55.
- HIEMSTRA, P. S., MAASSEN, R. J., STOLK, J., HEINZEL-WIELAND, R., STEFFENS, G. J. & DIJKMAN, J. H. 1996. Antibacterial activity of antileukoprotease. *Infect Immun*, 64, 4520-4.
- HIRANO, T., YASUKAWA, K., HARADA, H., TAGA, T., WATANABE, Y., MATSUDA, T., KASHIWAMURA, S., NAKAJIMA, K., KOYAMA, K., IWAMATSU, A. & ET AL. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature*, 324, 73-6.
- HIRCHE, T. O., ATKINSON, J. J., BAHR, S. & BELAAOUAJ, A. 2004. Deficiency in neutrophil elastase does not impair neutrophil recruitment to inflamed sites. *American journal of respiratory cell and molecular biology*, 30, 576-84.
- HIRSCH, J. G. 1958. Bactericidal action of histone. *The Journal of experimental medicine*, 108, 925-44.
- HOCHSTRASSER, K., ALBRECHT, G. J., SCHONBERGER, O. L., RASCHE, B. & LEMPART, K. 1981. An elastase-specific inhibitor from human bronchial mucus. Isolation and characterization. *Hoppe-Seyler's Zeitschrift fur physiologische Chemie*, 362, 1369-75.
- HOLLANDER, C., WESTIN, U., WALLMARK, A., PIITULAINEN, E., SVEGER, T. & JANCIAUSKIENE, S. M. 2007. Plasma levels of alpha1-antichymotrypsin and secretory leukocyte proteinase inhibitor in healthy and chronic obstructive pulmonary disease (COPD) subjects with and without severe alpha1-antitrypsin deficiency. *BMC pulmonary medicine*, 7, 1.
- HU, S., XIE, Y., RAMACHANDRAN, P., OGORZALEK LOO, R. R., LI, Y., LOO, J. A. & WONG, D. T. 2005. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. *Proteomics*, 5, 1714-28.
- HUGGETT, J., DHEDA, K., BUSTIN, S. & ZUMLA, A. 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes and immunity*, 6, 279-84.
- HUMPHREY, S. P. & WILLIAMSON, R. T. 2001a. A review of saliva: normal composition, flow, and function. *J Prosthet Dent*, 85, 162-9.

- HUMPHREY, S. P. & WILLIAMSON, R. T. 2001b. A review of saliva: normal composition, flow, and function. *The Journal of prosthetic dentistry*, 85, 162-9.
- IRELAND, R. 2006. *Clinical textbook of dental hygiene and therapy*, Oxford ; Ames, Iowa, Blackwell Munksgaard.
- ISAACSON, P., JONES, D. B., MILLWARD-SADLER, G. H., JUDD, M. A. & PAYNE, S. 1981. Alpha-1-antitrypsin in human macrophages. *Journal of clinical pathology*, 34, 982-90.
- JACOBSEN, L. C., SORENSEN, O. E., COWLAND, J. B., BORREGAARD, N. & THEILGAARD-MONCH, K. 2008. The secretory leukocyte protease inhibitor (SLPI) and the secondary granule protein lactoferrin are synthesized in myelocytes, colocalize in subcellular fractions of neutrophils, and are coreleased by activated neutrophils. *J Leukoc Biol*, 83, 1155-64.
- JANA, N. K., GRAY, L. R. & SHUGARS, D. C. 2005. Human immunodeficiency virus type I stimulates the expression and production of secretory leukocyte protease inhibitor (SLPI) in oral epithelial cells: a role for SLPI in innate mucosal immunity. *Journal of Virology*, 79, 6432-6440.
- JANOFF, A. 1972. Inhibition of human granulocyte elastase by serum alpha-1-antitrypsin. *The American review of respiratory disease*, 105, 121-2.
- JEPPSSON, J. O. & FRANZEN, B. 1982. Typing of genetic variants of alpha 1-antitrypsin by electrofocusing. *Clinical chemistry*, 28, 219-25.
- JIN, F. Y., NATHAN, C., RADZIOCH, D. & DING, A. 1997. Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. *Cell*, 88, 417-26.
- JUNQUEIRA, L. C., CARNEIRO, J. & KELLEY, R. O. 1995. *Basic histology*, London, Prentice-Hall International.
- KABURAKI, T., FUJINO, Y., KAWASHIMA, H., MERINO, G., NUMAGA, J., CHEN, J. & MATSUSHIMA, K. 2003. Plasma and whole-blood chemokine levels in patients with Behcet's disease. *Graefes Arch Clin Exp Ophthalmol*, 241, 353-8.
- KAKLAMANI, V. G., VAIPOULOS, G. & KAKLAMANIS, P. G. 1998. Behcet's Disease. *Semin Arthritis Rheum*, 27, 197-217.
- KALANTZIS, A., MARSHMAN, Z., FALCONER, D. T., MORGAN, P. R. & ODELL, E. W. 2005. Oral effects of low-dose methotrexate treatment. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics*, 100, 52-62.
- KAWASAKI, H. & IWAMURO, S. 2008. Potential roles of histones in host defense as antimicrobial agents. *Infectious disorders drug targets*, 8, 195-205.
- KEOGAN, M. T. 2009. Clinical Immunology Review Series: an approach to the patient with recurrent orogenital ulceration, including Behcet's syndrome. *Clinical and experimental immunology*, 156, 1-11.
- KESSENBROCK, K., DAU, T. & JENNE, D. E. 2011. Tailor-made inflammation: how neutrophil serine proteases modulate the inflammatory response. *J Mol Med (Berl)*, 89, 23-8.
- KESSENBROCK, K., KRUMBHOLZ, M., SCHONERMARCK, U., BACK, W., GROSS, W. L., WERB, Z., GRONE, H. J., BRINKMANN, V. & JENNE, D. E. 2009. Netting neutrophils in autoimmune small-vessel vasculitis. *Nature medicine*, 15, 623-5.
- KHANDPUR, R., CARMONA-RIVERA, C., VIVEKANANDAN-GIRI, A., GIZINSKI, A., YALAVARTHI, S., KNIGHT, J. S., FRIDAY, S., LI, S., PATEL, R. M., SUBRAMANIAN, V., THOMPSON, P., CHEN, P., FOX, D. A., PENNATHUR, S. & KAPLAN, M. J. 2013. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Science translational medicine*, 5, 178ra40.

- KIM, H. S., PARK, C. B., KIM, M. S. & KIM, S. C. 1996. cDNA cloning and characterization of buforin I, an antimicrobial peptide: a cleavage product of histone H2A. *Biochemical and biophysical research communications*, 229, 381-7.
- KIRCHEIS, R., MILLECK, J., KOROBKO, V. G., SHINGAROVA, L. N. & SCHMIDT, H. E. 1992. Differences in the biological activity of TNF alpha and TNF beta correlate with their different abilities for binding to the target cells. *European cytokine network*, 3, 381-90.
- KJELDSSEN, L., SENGELOV, H. & BORREGAARD, N. 1999. Subcellular fractionation of human neutrophils on Percoll density gradients. *Journal of immunological methods*, 232, 131-43.
- KNERER, B., FORMANEK, M., TEMMEL, A., MARTINEK, H., SCHICKINGER, B. & KORNFEHL, J. 1999. The role of fibroblasts from oropharyngeal mucosa in producing proinflammatory and mitogenic cytokines without prior stimulation. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies*, 256, 266-70.
- KOIZUMI, M., FUJINO, A., FUKUSHIMA, K., KAMIMURA, T. & TAKIMOTO-KAMIMURA, M. 2008. Complex of human neutrophil elastase with 1/2SLPI. *J Synchrotron Radiat*, 15, 308-11.
- KORKMAZ, B., ATTUCCI, S., JULIANO, M. A., KALUPOV, T., JOURDAN, M. L., JULIANO, L. & GAUTHIER, F. 2008a. Measuring elastase, proteinase 3 and cathepsin G activities at the surface of human neutrophils with fluorescence resonance energy transfer substrates. *Nature protocols*, 3, 991-1000.
- KORKMAZ, B., MOREAU, T. & GAUTHIER, F. 2008b. Neutrophil elastase, proteinase 3 and cathepsin G: physicochemical properties, activity and physiopathological functions. *Biochimie*, 90, 227-42.
- KOSE, O., STEWART, J., WASEEM, A., LALLI, A. & FORTUNE, F. 2008. Expression of cytokeratins, adhesion and activation molecules in oral ulcers of Behcet's disease. *Clinical and experimental dermatology*, 33, 62-9.
- KROWARSCH, D., CIERPICKI, T., JELEN, F. & OTLEWSKI, J. 2003. Canonical protein inhibitors of serine proteases. *Cell Mol Life Sci*, 60, 2427-44.
- KUMAR, B. P., KELUSKAR, V., BAGEWADI, A. S. & SHETTI, A. 2010. Evaluating and comparing phagocytic functions of salivary and blood neutrophils in patients with recurrent aphthous ulcers and controls. *Quintessence international*, 41, 411-6.
- KURAL-SEYAHİ, E., FRESKO, I., SEYAHİ, N., OZYAZGAN, Y., MAT, C., HAMURYUDAN, V., YURDAKUL, S. & YAZICI, H. 2003. The long-term mortality and morbidity of Behcet syndrome: a 2-decade outcome survey of 387 patients followed at a dedicated center. *Medicine*, 82, 60-76.
- KURAUCHI, T., YOKOTA, K., MATSUO, T., FUJINAMI, Y., ISOGAI, E., ISOGAI, H., OHTSUKI, H. & OGUMA, K. 2005. Neutrophil and lymphocyte responses to oral Streptococcus in Adamantiades-Behcet's disease. *FEMS immunology and medical microbiology*, 43, 125-31.
- KUROKAWA, M. S., YOSHIKAWA, H. & SUZUKI, N. 2004. Behcet's disease. *Seminars in Respiratory and Critical Care Medicine*, 25, 557-568.
- LACY, P. 2006. Mechanisms of degranulation in neutrophils. *Allergy Asthma Clin Immunol*, 2, 98-108.
- LAKSCHEVITZ, F. S., ABOODI, G. M. & GLOGAUER, M. 2013. Oral neutrophil transcriptome changes result in a pro-survival phenotype in periodontal diseases. *PLoS One*, 8, e68983.
- LE-BARILLEC, K., SI-TAHAR, M., BALLOY, V. & CHIGNARD, M. 1999. Proteolysis of monocyte CD14 by human leukocyte elastase inhibits

- lipopolysaccharide-mediated cell activation. *The Journal of clinical investigation*, 103, 1039-46.
- LEE, C. H., IGARASHI, Y., HOHMAN, R. J., KAULBACH, H., WHITE, M. V. & KALINER, M. A. 1993. Distribution of Secretory Leukoprotease Inhibitor in the Human Nasal Airway. *American Review of Respiratory Disease*, 147, 710-716.
- LEE, S. K., LEE, S. S., HIROSE, S., PARK, S. C., CHI, J. G., CHUNG, S. I. & MORI, M. 2002. Elafin expression in human fetal and adult submandibular glands. *Histochem Cell Biol*, 117, 423-30.
- LEE, W. L. & DOWNEY, G. P. 2001. Leukocyte elastase: physiological functions and role in acute lung injury. *American journal of respiratory and critical care medicine*, 164, 896-904.
- LEE, Y. H. & WONG, D. T. 2009. Saliva: an emerging biofluid for early detection of diseases. *American journal of dentistry*, 22, 241-8.
- LEHNER, T. 1978. Immunological aspects of recurrent oral ulceration and Behcet's syndrome. *Journal of oral pathology*, 7, 424-30.
- LEVY, Y. & BROUET, J. C. 1994. Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein. *The Journal of clinical investigation*, 93, 424-8.
- LEWKOWICZ, N., LEWKOWICZ, P., KURNATOWSKA, A., BANASIK, M., GLOWACKA, E., CEDZYNSKI, M., SWIERZKO, A., LAUK-PUCHALA, B. & TCHORZEWSKI, H. 2003. Innate immune system is implicated in recurrent aphthous ulcer pathogenesis. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 32, 475-81.
- LEY, K. 2002. Integration of inflammatory signals by rolling neutrophils. *Immunological reviews*, 186, 8-18.
- LI, Z., MOY, A., SOHAL, K., DAM, C., KUO, P., WHITTAKER, J., WHITTAKER, M., DUZGUNES, N., KONOPKA, K., FRANZ, A. H., LIN-CEREGHINO, J. & LIN-CEREGHINO, G. P. 2009. Expression and characterization of recombinant human secretory leukocyte protease inhibitor (SLPI) protein from *Pichia pastoris*. *Protein Expr Purif*, 67, 175-81.
- LIU, T. G. & CAMPBELL, E. J. 1995. Nonisotropic enzyme--inhibitor interactions: a novel nonoxidative mechanism for quantum proteolysis by human neutrophils. *Biochemistry*, 34, 16171-7.
- LOGTERS, T., MARGRAF, S., ALTRICHTER, J., CINATL, J., MITZNER, S., WINDOLF, J. & SCHOLZ, M. 2009. The clinical value of neutrophil extracellular traps. *Medical microbiology and immunology*, 198, 211-9.
- LUCAS, S. D., COSTA, E., GUEDES, R. C. & MOREIRA, R. 2011. Targeting COPD: Advances on low-molecular-weight inhibitors of human neutrophil elastase. *Med Res Rev*.
- LUCEY, D. R., CLERICI, M. & SHEARER, G. M. 1996. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clinical microbiology reviews*, 9, 532-62.
- LUKAC, J., MRAVAK-STIPETIC, M., KNEZEVIC, M., VRCEK, J., SISTIG, S., LEDINSKY, M. & KUSIC, Z. 2003. Phagocytic functions of salivary neutrophils in oral mucous membrane diseases. *J Oral Pathol Med*, 32, 271-4.
- LUNGARELLA, G., MENEGAZZI, R., GARDI, C., SPESSOTTO, P., DE SANTI, M. M., BERTONCIN, P., PATRIARCA, P., CALZONI, P. & ZABUCCHI, G. 1992. Identification of elastase in human eosinophils: immunolocalization, isolation, and partial characterization. *Archives of biochemistry and biophysics*, 292, 128-35.
- MA, A. C. & KUBES, P. 2008. Platelets, neutrophils, and neutrophil extracellular traps (NETs) in sepsis. *Journal of thrombosis and haemostasis : JTH*, 6, 415-20.

- MAIN, D. M. & CHAMBERLAIN, M. A. 1992. Clinical differentiation of oral ulceration in Behcet's disease. *Br J Rheumatol*, 31, 767-70.
- MAIZIERES, M., KAPLAN, H., MILLOT, J. M., BONNET, N., MANFAIT, M., PUCHELLE, E. & JACQUOT, J. 1998. Neutrophil elastase promotes rapid exocytosis in human airway gland cells by producing cytosolic Ca²⁺ oscillations. *Am J Respir Cell Mol Biol*, 18, 32-42.
- MALLE, E., FURTMULLER, P. G., SATTLER, W. & OBINGER, C. 2007. Myeloperoxidase: a target for new drug development? *British journal of pharmacology*, 152, 838-54.
- MALTZMAN, J. S. & KORETZKY, G. A. 2003. Azathioprine: old drug, new actions. *The Journal of clinical investigation*, 111, 1122-4.
- MANTAS, C., DIRESKENELI, H., OZ, D., YAVUZ, S. & AKOGLU, T. 2000. IL-8 producing cells in patients with Behcet's disease. *Clinical and experimental rheumatology*, 18, 249-51.
- MANTHEY, H. D., WOODRUFF, T. M., TAYLOR, S. M. & MONK, P. N. 2009. Complement component 5a (C5a). *The international journal of biochemistry & cell biology*, 41, 2114-7.
- MARSH, P. D. 2012. Contemporary perspective on plaque control. *British dental journal*, 212, 601-6.
- MARSHALL, S. E. 2004. Behcet's disease. *Best Pract Res Clin Rheumatol*, 18, 291-311.
- MASON, D. Y., CRAMER, E. M., MASSE, J. M., CRYSTAL, R., BASSOT, J. M. & BRETON-GORIUS, J. 1991. Alpha 1-antitrypsin is present within the primary granules of human polymorphonuclear leukocytes. *The American journal of pathology*, 139, 623-8.
- MATHESON, N. R., WONG, P. S. & TRAVIS, J. 1979. Enzymatic inactivation of human alpha-1-proteinase inhibitor by neutrophil myeloperoxidase. *Biochem Biophys Res Commun*, 88, 402-9.
- MATSUMURA, N. & MIZUSHIMA, Y. 1975. Leucocyte movement and colchicine treatment in Behcet's disease. *Lancet*, 2, 813.
- MCELVANEY, N. G., DOUJAJI, B., MOAN, M. J., BURNHAM, M. R., WU, M. C. & CRYSTAL, R. G. 1993. Pharmacokinetics of recombinant secretory leukoprotease inhibitor aerosolized to normals and individuals with cystic fibrosis. *The American review of respiratory disease*, 148, 1056-60.
- MCELVANEY, N. G., NAKAMURA, H., BIRRER, P., HEBERT, C. A., WONG, W. L., ALPHONSO, M., BAKER, J. B., CATALANO, M. A. & CRYSTAL, R. G. 1992. Modulation of airway inflammation in cystic fibrosis. In vivo suppression of interleukin-8 levels on the respiratory epithelial surface by aerosolization of recombinant secretory leukoprotease inhibitor. *The Journal of clinical investigation*, 90, 1296-301.
- MCNEELY, T. B., DEALY, M., DRIPPS, D. J., ORENSTEIN, J. M., EISENBERG, S. P. & WAHL, S. M. 1995. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. *The Journal of clinical investigation*, 96, 456-64.
- MECHAM, R. P., BROEKELMANN, T. J., FLISZAR, C. J., SHAPIRO, S. D., WELGUS, H. G. & SENIOR, R. M. 1997. Elastin degradation by matrix metalloproteinases. Cleavage site specificity and mechanisms of elastolysis. *The Journal of biological chemistry*, 272, 18071-6.
- MEGE, J. L., DILSEN, N., SANGUEDOLCE, V., GUL, A., BONGRAND, P., ROUX, H., OCAL, L., INANC, M. & CAPO, C. 1993. Overproduction of monocyte derived tumor necrosis factor alpha, interleukin (IL) 6, IL-8 and increased neutrophil superoxide generation in Behcet's disease. A comparative study with

- familial Mediterranean fever and healthy subjects. *The Journal of rheumatology*, 20, 1544-9.
- MELLETT, M., ATZEI, P., HORGAN, A., HAMS, E., FLOSS, T., WURST, W., FALLON, P. G. & MOYNAGH, P. N. 2012. Orphan receptor IL-17RD tunes IL-17A signalling and is required for neutrophilia. *Nature communications*, 3, 1119.
- MENDES, D., CORREIA, M., BARBEDO, M., VAIO, T., MOTA, M., GONCALVES, O. & VALENTE, J. 2009. Behcet's disease--a contemporary review. *J Autoimmun*, 32, 178-88.
- MESQUITA JR, D., CRUVINEL, W. M., CAMARA, N. O., KALLAS, E. G. & ANDRADE, L. E. 2009. Autoimmune diseases in the TH17 era. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.]*, 42, 476-86.
- METZLER, K. D., FUCHS, T. A., NAUSEEF, W. M., REUMAUX, D., ROESLER, J., SCHULZE, I., WAHN, V., PAPAYANNOPOULOS, V. & ZYCHLINSKY, A. 2011. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood*, 117, 953-9.
- MICHAELIS, J., VISSERS, M. C. & WINTERBOURN, C. C. 1990. Human neutrophil collagenase cleaves alpha 1-antitrypsin. *The Biochemical journal*, 270, 809-14.
- MILLER, C. S., FOLEY, J. D., BAILEY, A. L., CAMPELL, C. L., HUMPHRIES, R. L., CHRISTODOULIDES, N., FLORIANO, P. N., SIMMONS, G., BHAGWANDIN, B., JACOBSON, J. W., REDDING, S. W., EBERSOLE, J. L. & MCDEVITT, J. T. 2010. Current developments in salivary diagnostics. *Biomarkers in medicine*, 4, 171-89.
- MIRAVITLES, M. 2012. Alpha-1-antitrypsin and other proteinase inhibitors. *Curr Opin Pharmacol*, 12, 309-14.
- MIRZAI-DIZGAH, I. & RIAHI, E. 2011. Serum and saliva levels of cathepsin L in patients with acute coronary syndrome. *The journal of contemporary dental practice*, 12, 114-9.
- MOHARAMZADEH, K., BROOK, I. M., VAN NOORT, R., SCUTT, A. M. & THORNHILL, M. H. 2007. Tissue-engineered oral mucosa: a review of the scientific literature. *J Dent Res*, 86, 115-24.
- MOREAU, T., BARANGER, K., DADE, S., DALLEY-CHOISY, S., GUYOT, N. & ZANI, M. L. 2008. Multifaceted roles of human elafin and secretory leukocyte proteinase inhibitor (SLPI), two serine protease inhibitors of the chelonianin family. *Biochimie*, 90, 284-95.
- MORGAN, K., CHAPPELL, S., GUETTA-BARANES, T., MORLEY, S. & KALSHEKER, N. 2009. The alpha-1-antitrypsin gene promoter in human A549 lung derived cells, and a novel transcription initiation site. *The international journal of biochemistry & cell biology*, 41, 1157-64.
- MORIYAMA, A., SHIMOYA, K., OGATA, I., KIMURA, T., NAKAMURA, T., WADA, H., OHASHI, K., AZUMA, C., SAJI, F. & MURATA, Y. 1999. Secretory leukocyte protease inhibitor (SLPI) concentrations in cervical mucus of women with normal menstrual cycle. *Molecular Human Reproduction*, 5, 656-661.
- MORNEX, J. F., CHYTIL-WEIR, A., MARTINET, Y., COURTNEY, M., LECOCQ, J. P. & CRYSTAL, R. G. 1986. Expression of the alpha-1-antitrypsin gene in mononuclear phagocytes of normal and alpha-1-antitrypsin-deficient individuals. *The Journal of clinical investigation*, 77, 1952-61.
- MOROHASHI, H., MIYAWAKI, T., NOMURA, H., KUNO, K., MURAKAMI, S., MATSUSHIMA, K. & MUKAIDA, N. 1995. Expression of both types of human interleukin-8 receptors on mature neutrophils, monocytes, and natural killer cells. *Journal of leukocyte biology*, 57, 180-7.

- MORRISON, H. M., WELGUS, H. G., STOCKLEY, R. A., BURNETT, D. & CAMPBELL, E. J. 1990. Inhibition of human leukocyte elastase bound to elastin: relative ineffectiveness and two mechanisms of inhibitory activity. *Am J Respir Cell Mol Biol*, 2, 263-9.
- MUMCU, G. 2004. Oral health is impaired in Behcet's disease and is associated with disease severity. *Rheumatology*, 43, 1028-1033.
- MUMCU, G., HAYRAN, O., OZALP, D. O., INANC, N., YAVUZ, S., ERGUN, T. & DIRESKENELI, H. 2007. The assessment of oral health-related quality of life by factor analysis in patients with Behcet's disease and recurrent aphthous stomatitis. *Journal of Oral Pathology & Medicine*, 36, 147-152.
- MUMCU, G., NIAZI, S., STEWART, J., HAGI-PAVLI, E., GOKANI, B., SEOUDI, N., ERGUN, T., YAVUZ, S., STANFORD, M., FORTUNE, F. & DIRESKENELI, H. 2009. Oral health and related quality of life status in patients from UK and Turkey: a comparative study in Behcet's disease. *J Oral Pathol Med*, 38, 406-9.
- MUNOZ-CORCUERA, M., ESPARZA-GOMEZ, G., GONZALEZ-MOLES, M. A. & BASCONES-MARTINEZ, A. 2009. Oral ulcers: clinical aspects. A tool for dermatologists. Part II. Chronic ulcers. *Clin Exp Dermatol*, 34, 456-61.
- MURDOCH, C., MONK, P. N. & FINN, A. 1999. Cxc chemokine receptor expression on human endothelial cells. *Cytokine*, 11, 704-12.
- MURPHY, P. M. & TIFFANY, H. L. 1991. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science*, 253, 1280-3.
- NAKA, T., NISHIMOTO, N. & KISHIMOTO, T. 2002. The paradigm of IL-6: from basic science to medicine. *Arthritis research*, 4 Suppl 3, S233-42.
- NAKAHARA, H., SATO, E. F., ISHISAKA, R., KANNO, T., YOSHIOKA, T., YASUDA, T., INOUE, M. & UTSUMI, K. 1998. Biochemical properties of human oral polymorphonuclear leukocytes. *Free radical research*, 28, 485-95.
- NAKAMURA-MINAMI, M., FURUICHI, Y., ISHIKAWA, K., MITSUZONO-TOFUKU, Y. & IZUMI, Y. 2003. Changes of alpha1-protease inhibitor and secretory leukocyte protease inhibitor levels in gingival crevicular fluid before and after non-surgical periodontal treatment. *Oral diseases*, 9, 249-54.
- NAPIREI, M., WULF, S. & MANNHERZ, H. G. 2004. Chromatin breakdown during necrosis by serum Dnase1 and the plasminogen system. *Arthritis and rheumatism*, 50, 1873-83.
- NATAH, S. S., HAYRINEN-IMMONEN, R., HIETANEN, J., PATINEN, P., MALMSTROM, M., SAVILAHTI, E. & KONTTINEN, Y. T. 2000. Increased density of lymphocytes bearing gamma/delta T-cell receptors in recurrent aphthous ulceration (RAU). *International journal of oral and maxillofacial surgery*, 29, 375-80.
- NATAH, S. S., KONTTINEN, Y. T., ENATTAH, N. S., ASHAMMAKHI, N., SHARKEY, K. A. & HAYRINEN-IMMONEN, R. 2004. Recurrent aphthous ulcers today: a review of the growing knowledge. *Int J Oral Maxillofac Surg*, 33, 221-34.
- NATHAN, C. 2006. Neutrophils and immunity: challenges and opportunities. *Nature reviews. Immunology*, 6, 173-82.
- NEMOTO, E., SUGAWARA, S., TADA, H., TAKADA, H., SHIMAUCHI, H. & HORIUCHI, H. 2000. Cleavage of CD14 on human gingival fibroblasts cocultured with activated neutrophils is mediated by human leukocyte elastase resulting in down-regulation of lipopolysaccharide-induced IL-8 production. *Journal of immunology*, 165, 5807-13.
- NEVES, F. S., CARRASCO, S., GOLDENSTEIN-SCHAINBERG, C., GONCALVES, C. R. & DE MELLO, S. B. 2009. Neutrophil hyperchemotaxis in Behcet's disease:

- a possible role for monocytes orchestrating bacterial-induced innate immune responses. *Clinical rheumatology*, 28, 1403-10.
- NITTAYANANTA, W., KEMAPUNMANUS, M., YANGNGAM, S., TALUNGCHIT, S. & SRIPLUNG, H. 2013a. Expression of oral secretory leukocyte protease inhibitor in HIV-infected subjects with long-term use of antiretroviral therapy. *J Oral Pathol Med*, 42, 208-15.
- NITTAYANANTA, W., KEMAPUNMANUS, M., YANGNGAM, S., TALUNGCHIT, S. & SRIPLUNG, H. 2013b. Expression of oral secretory leukocyte protease inhibitor in HIV-infected subjects with long-term use of antiretroviral therapy. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 42, 208-15.
- NOLAN, T., HANDS, R. E. & BUSTIN, S. A. 2006a. Quantification of mRNA using real-time RT-PCR. *Nature protocols*, 1, 1559-82.
- NOLAN, T., HANDS, R. E., OGUNKOLADE, W. & BUSTIN, S. A. 2006b. SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. *Analytical biochemistry*, 351, 308-10.
- NYSTROM, M., BERGENFELDT, M., LJUNGCRANTZ, I., LINDEHEIM, A. & OHLSSON, K. 1999. Production of secretory leukocyte protease inhibitor (SLPI) in human pancreatic beta-cells. *Mediators of inflammation*, 8, 147-51.
- O'DONOGHUE, A. J., JIN, Y., KNUDSEN, G. M., PERERA, N. C., JENNE, D. E., MURPHY, J. E., CRAIK, C. S. & HERMISTON, T. W. 2013. Global substrate profiling of proteases in human neutrophil extracellular traps reveals consensus motif predominantly contributed by elastase. *PLoS One*, 8, e75141.
- O'DUFFY, J. D., LEHNER, T. & BARNES, C. G. 1983. Summary of the Third International Conference on Behcet's Disease, Tokyo, Japan, October 23-24, 1981. *The Journal of rheumatology*, 10, 154-8.
- OH, S. H., HAN, E. C., LEE, J. H. & BANG, D. 2009. Comparison of the clinical features of recurrent aphthous stomatitis and Behcet's disease. *Clin Exp Dermatol*, 34, e208-12.
- OHLSSON, K., BJARTELL, A. & LILJA, H. 1995. Secretory Leukocyte Protease Inhibitor in the Male Genital-Tract - Psa-Induced Proteolytic Processing in Human Semen and Tissue Localization. *Journal of Andrology*, 16, 64-74.
- OHLSSON, M., FRYKSMARK, U., POLLING, A., TEGNER, H. & OHLSSON, K. 1984. Localization of antileukoprotease in the parotid and the submandibular salivary glands. *Acta oto-laryngologica*, 98, 147-51.
- OHLSSON, S., TUFVESSON, B., POLLING, A. & OHLSSON, K. 1997. Distribution of the secretory leukocyte proteinase inhibitor in human articular cartilage. *Biological chemistry*, 378, 1055-8.
- OKADA, A. A., GOTO, H., OHNO, S. & MOCHIZUKI, M. 2012. Multicenter study of infliximab for refractory uveoretinitis in Behcet disease. *Archives of ophthalmology*, 130, 592-8.
- OKAZAKI, M., YOSHIMURA, K., SUZUKI, Y. & HARII, K. 2003. Effects of subepithelial fibroblasts on epithelial differentiation in human skin and oral mucosa: heterotypically recombined organotypic culture model. *Plast Reconstr Surg*, 112, 784-92.
- OVERBERGH, L., GIULIETTI, A., VALCKX, D., DECALLONNE, R., BOUILLON, R. & MATHIEU, C. 2003. The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *Journal of biomolecular techniques : JBT*, 14, 33-43.
- OWEN, C. A., CAMPBELL, M. A., BOUKEDES, S. S. & CAMPBELL, E. J. 1997. Cytokines regulate membrane-bound leukocyte elastase on neutrophils: a novel

- mechanism for effector activity. *The American journal of physiology*, 272, L385-93.
- OWEN, C. A., CAMPBELL, M. A., SANNES, P. L., BOUKEDES, S. S. & CAMPBELL, E. J. 1995. Cell surface-bound elastase and cathepsin G on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *The Journal of cell biology*, 131, 775-89.
- OZCAKA, O., NALBANTSOY, A. & BUDUNELI, N. 2011. Interleukin-17 and interleukin-18 levels in saliva and plasma of patients with chronic periodontitis. *Journal of periodontal research*, 46, 592-8.
- OZDAMAR, Y., BERKER, N., BAHAR, G., SOYKAN, E., BICER, T., OZKAN, S. S. & KARAKAYA, J. 2009. Inflammatory mediators and posterior segment involvement in ocular Behcet disease. *European journal of ophthalmology*, 19, 998-1003.
- PAAKKO, P., KIRBY, M., DU BOIS, R. M., GILLISSEN, A., FERRANS, V. J. & CRYSTAL, R. G. 1996. Activated neutrophils secrete stored alpha 1-antitrypsin. *American journal of respiratory and critical care medicine*, 154, 1829-33.
- PAPAYANNOPOULOS, V., METZLER, K. D., HAKKIM, A. & ZYCHLINSKY, A. 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *The Journal of cell biology*, 191, 677-91.
- PAPAYANNOPOULOS, V., STAAB, D. & ZYCHLINSKY, A. 2011. Neutrophil elastase enhances sputum solubilization in cystic fibrosis patients receiving DNase therapy. *PLoS One*, 6, e28526.
- PAPAYANNOPOULOS, V. & ZYCHLINSKY, A. 2009. NETs: a new strategy for using old weapons. *Trends in immunology*, 30, 513-21.
- PARKER, H., DRAGUNOW, M., HAMPTON, M. B., KETTLE, A. J. & WINTERBOURN, C. C. 2012. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *Journal of leukocyte biology*, 92, 841-9.
- PEDERSON, E. D., STANKE, S. R., WHITENER, S. J., SEBASTIANI, P. T., LAMBERTS, B. L. & TURNER, D. W. 1995. Salivary levels of alpha 2-macroglobulin, alpha 1-antitrypsin, C-reactive protein, cathepsin G and elastase in humans with or without destructive periodontal disease. *Archives of oral biology*, 40, 1151-5.
- PEPIN, L. F., ROGER, T., MORISSET, J. & SEMAN, M. 1993. Preferential V delta 1 expression among TcR gamma/delta-bearing T cells in human oral epithelium. *Scandinavian journal of immunology*, 37, 289-94.
- PERANTEAU, W. H., ZHANG, L., MUVARAK, N., BADILLO, A. T., RADU, A., ZOLTICK, P. W. & LIECHTY, K. W. 2008. IL-10 overexpression decreases inflammatory mediators and promotes regenerative healing in an adult model of scar formation. *The Journal of investigative dermatology*, 128, 1852-60.
- PERLMUTTER, D. H., COLE, F. S., KILBRIDGE, P., ROSSING, T. H. & COLTEN, H. R. 1985. Expression of the alpha 1-proteinase inhibitor gene in human monocytes and macrophages. *Proceedings of the National Academy of Sciences of the United States of America*, 82, 795-9.
- PICCIONI, P. D., KRAMPS, J. A., RUDOLPHUS, A., BULGHERONI, A. & LUISETTI, M. 1992. Proteinase/proteinase inhibitor imbalance in sputum sol phases from patients with chronic obstructive pulmonary disease. Suggestions for a key role played by antileukoprotease. *Chest*, 102, 1470-6.
- PILETTE, C., OUADRHIRI, Y., DIMANCHE, F., VAERMAN, J. P. & SIBILLE, Y. 2003. Secretory component is cleaved by neutrophil serine proteinases but its epithelial production is increased by neutrophils through NF-kappa B- and p38

- mitogen-activated protein kinase-dependent mechanisms. *American journal of respiratory cell and molecular biology*, 28, 485-98.
- PILLAY, J., DEN BRABER, I., VRISEKOOP, N., KWAST, L. M., DE BOER, R. J., BORGHANS, J. A., TESSELAAR, K. & KOENDERMAN, L. 2010. In vivo labeling with $2\text{H}_2\text{O}$ reveals a human neutrophil lifespan of 5.4 days. *Blood*, 116, 625-7.
- QUINN, D. J., WELDON, S. & TAGGART, C. C. 2010. Antiproteases as therapeutics to target inflammation in cystic fibrosis. *Open Respir Med J*, 4, 20-31.
- RAIFER, H., MAHINY, A. J., BOLLIG, N., PETERMANN, F., HELLHUND, A., KELLNER, K., GURALNIK, A., REINHARD, K., BOTHUR, E., HUBER, M., BAUER, S., LOHNING, M., KISS, E. A., GANAL, S. C., DIEFENBACH, A., KORN, T. & LOHOFF, M. 2012. Unlike alphabeta T cells, gammadelta T cells, LT α cells and NKT cells do not require IRF4 for the production of IL-17A and IL-22. *European journal of immunology*, 42, 3189-201.
- REIBETANZ, U., SCHONBERG, M., RATHMANN, S., STREHLOW, V., GOSE, M. & LESSIG, J. 2012. Inhibition of human neutrophil elastase by alpha1-antitrypsin functionalized colloidal microcarriers. *ACS nano*, 6, 6325-36.
- REILLY, C. F. & TRAVIS, J. 1980. The degradation of human lung elastin by neutrophil proteinases. *Biochim Biophys Acta*, 621, 147-57.
- RENTOFT, M., HULTIN, S., COATES, P. J., LAURELL, G. & NYLANDER, K. 2010. Tubulin alpha-6 chain is a stably expressed reference gene in archival samples of normal oral tissue and oral squamous cell carcinoma. *Experimental and therapeutic medicine*, 1, 419-423.
- REVUZ, J., GUILLAUME, J. C., JANIER, M., HANS, P., MARCHAND, C., SOUTEYRAND, P., BONNETBLANC, J. M., CLAUDY, A., DALLAC, S., KLENE, C. & ET AL. 1990. Crossover study of thalidomide vs placebo in severe recurrent aphthous stomatitis. *Archives of dermatology*, 126, 923-7.
- RIEMER, A. B., KESKIN, D. B. & REINHERZ, E. L. 2012. Identification and validation of reference genes for expression studies in human keratinocyte cell lines treated with and without interferon-gamma - a method for qRT-PCR reference gene determination. *Experimental dermatology*, 21, 625-9.
- RIPPLE, M. O. & WILDING, G. 1995. Alteration of glyceraldehyde-3-phosphate dehydrogenase activity and messenger RNA content by androgen in human prostate carcinoma cells. *Cancer research*, 55, 4234-6.
- ROBERTSON, L. P. & HICKLING, P. 2001. Treatment of recalcitrant orogenital ulceration of Behcet's syndrome with infliximab. *Rheumatology*, 40, 473-4.
- ROGHANIAN, A. & SALLENAVE, J. M. 2008a. Neutrophil elastase (NE) and NE inhibitors: canonical and noncanonical functions in lung chronic inflammatory diseases (cystic fibrosis and chronic obstructive pulmonary disease). *Journal of aerosol medicine and pulmonary drug delivery*, 21, 125-44.
- ROGHANIAN, A. & SALLENAVE, J. M. 2008b. Neutrophil elastase (NE) and NE inhibitors: canonical and noncanonical functions in lung chronic inflammatory diseases (cystic fibrosis and chronic obstructive pulmonary disease). *J Aerosol Med Pulm Drug Deliv*, 21, 125-44.
- ROMAGNANI, S. 1995. Biology of human TH1 and TH2 cells. *Journal of clinical immunology*, 15, 121-9.
- ROSENBERG, S., BARR, P. J., NAJARIAN, R. C. & HALLEWELL, R. A. 1984. Synthesis in yeast of a functional oxidation-resistant mutant of human alpha-antitrypsin. *Nature*, 312, 77-80.
- ROUBILLE, F., KRITIKOU, E., BUSSEUIL, D., BARRERE-LEMAIRE, S. & TARDIF, J. C. 2013. Colchicine: an old wine in a new bottle? *Anti-inflammatory & anti-allergy agents in medicinal chemistry*, 12, 14-23.

- RUDOLPHUS, A., HEINZEL-WIELAND, R., VINCENT, V. A., SAUNDERS, D., STEFFENS, G. J., DIJMAN, J. H. & KRAMPS, J. A. 1991. Oxidation-resistant variants of recombinant antileucoprotease are better inhibitors of human-neutrophil-elastase-induced emphysema in hamsters than natural recombinant antileucoprotease. *Clinical science*, 81, 777-84.
- SABROE, I. & WHYTE, M. K. 2007. Toll-like receptor (TLR)-based networks regulate neutrophilic inflammation in respiratory disease. *Biochemical Society transactions*, 35, 1492-5.
- SAFFARZADEH, M., JUENEMANN, C., QUEISSER, M. A., LOCHNIT, G., BARRETO, G., GALUSKA, S. P., LOHMEYER, J. & PREISSNER, K. T. 2012. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS One*, 7, e32366.
- SAHIN, S., AKOGLU, T., DIRESKENELI, H., SEN, L. S. & LAWRENCE, R. 1996. Neutrophil adhesion to endothelial cells and factors affecting adhesion in patients with Behcet's disease. *Annals of the rheumatic diseases*, 55, 128-33.
- SAITOH, H., MASUDA, T., SHIMURA, S., FUSHIMI, T. & SHIRATO, K. 2001. Secretion and gene expression of secretory leukocyte protease inhibitor by human airway submucosal glands. *American journal of physiology. Lung cellular and molecular physiology*, 280, L79-87.
- SAKANE, T., TAKENO, M., SUZUKI, N. & INABA, G. 1999. Behcet's disease. *The New England journal of medicine*, 341, 1284-91.
- SALLENAVE, J. M. 2000. The role of secretory leukocyte proteinase inhibitor and elafin (elastase-specific inhibitor/skin-derived antileukoprotease) as alarm antiproteases in inflammatory lung disease. *Respir Res*, 1, 87-92.
- SALLENAVE, J. M., SHULMANN, J., CROSSLEY, J., JORDANA, M. & GAULDIE, J. 1994. Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. *Am J Respir Cell Mol Biol*, 11, 733-41.
- SALLENAVE, J. M., SI TAHAR, M., COX, G., CHIGNARD, M. & GAULDIE, J. 1997. Secretory leukocyte proteinase inhibitor is a major leukocyte elastase inhibitor in human neutrophils. *J Leukoc Biol*, 61, 695-702.
- SANDER, H. M. & RANDLE, H. W. 1986. Use of colchicine in Behcet's syndrome. *Cutis*, 37, 344-8.
- SATHE, S., SAKATA, M., BEATON, A. R. & SACK, R. A. 1998. Identification, origins and the diurnal role of the principal serine protease inhibitors in human tear fluid. *Current Eye Research*, 17, 348-362.
- SCHINDELIN, J., ARGANDA-CARRERAS, I., FRISE, E., KAYNIG, V., LONGAIR, M., PIETZSCH, T., PREIBISCH, S., RUEDEN, C., SAALFELD, S., SCHMID, B., TINEVEZ, J. Y., WHITE, D. J., HARTENSTEIN, V., ELICEIRI, K., TOMANCAK, P. & CARDONA, A. 2012. Fiji: an open-source platform for biological-image analysis. *Nature methods*, 9, 676-82.
- SCHIPPER, R. G., SILLETTI, E. & VINGERHOEDS, M. H. 2007. Saliva as research material: biochemical, physicochemical and practical aspects. *Archives of oral biology*, 52, 1114-35.
- SCHMID, M., FELLERMANN, K., FRITZ, P., WIEDOW, O., STANGE, E. F. & WEHKAMP, J. 2007. Attenuated induction of epithelial and leukocyte serine antiproteases elafin and secretory leukocyte protease inhibitor in Crohn's disease. *Journal of leukocyte biology*, 81, 907-15.
- SCHONBERG, M., REIBETANZ, U., RATHMANN, S. & LESSIG, J. 2012. Maintenance of alpha(1)-antitrypsin activity by means of co-application of hypochlorous acid-scavengers in vitro and in the supernatant of

- polymorphonuclear leukocytes: as a basis for a new drug delivery approach. *Biomatter*, 2, 24-36.
- SCHRAUFSTATTER, I. U., CHUNG, J. & BURGER, M. 2001. IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways. *American journal of physiology. Lung cellular and molecular physiology*, 280, L1094-103.
- SCULLY, C. & FELIX, D. H. 2005. Oral medicine--update for the dental practitioner. Aphthous and other common ulcers. *British dental journal*, 199, 259-64.
- SCULLY, C. & PORTER, S. 2008. Oral mucosal disease: recurrent aphthous stomatitis. *Br J Oral Maxillofac Surg*, 46, 198-206.
- SENGELOV, H., FOLLIN, P., KJELDSEN, L., LOLLIKE, K., DAHLGREN, C. & BORREGAARD, N. 1995. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. *J Immunol*, 154, 4157-65.
- SENGELOV, H., KJELDSEN, L. & BORREGAARD, N. 1993. Control of exocytosis in early neutrophil activation. *J Immunol*, 150, 1535-43.
- SENSI, A., GAVIOLI, R., SPISANI, S., BALBONI, A., MELCHIORRI, L., MENICUCCI, A., PALUMBO, G., TRANIELLO, S. & BARICORDI, O. R. 1991. HLA B51 antigen associated with neutrophil hyper-reactivity. *Disease markers*, 9, 327-31.
- SEYAH, E. & YURDAKUL, S. 2011. Behcet's Syndrome and Thrombosis. *Mediterranean journal of hematology and infectious diseases*, 3, e2011026.
- SHETH, K. V., ALKAFF, M. A., BAHABRI, S. A., EL RAMAHI, K. M., AL-SEDAIRY, S. & AL-DALAN, A. A. 1995. Evaluation of anti-ds DNA antibody measurement by using commercial kits for use in a clinical laboratory. *Annals of Saudi medicine*, 15, 327-32.
- SHI, G. P., MUNGER, J. S., MEARA, J. P., RICH, D. H. & CHAPMAN, H. A. 1992. Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastolytic cysteine protease. *The Journal of biological chemistry*, 267, 7258-62.
- SHI, G. P., WEBB, A. C., FOSTER, K. E., KNOLL, J. H., LEMERE, C. A., MUNGER, J. S. & CHAPMAN, H. A. 1994. Human cathepsin S: chromosomal localization, gene structure, and tissue distribution. *The Journal of biological chemistry*, 269, 11530-6.
- SHIMIZU, J., TAKAI, K., FUJIWARA, N., ARIMITSU, N., UEDA, Y., WAKISAKA, S., YOSHIKAWA, H., KANEKO, F., SUZUKI, T. & SUZUKI, N. 2012. Excessive CD4+ T cells co-expressing interleukin-17 and interferon-gamma in patients with Behcet's disease. *Clinical and experimental immunology*, 168, 68-74.
- SHOJAEI, A. H. 1998. Buccal mucosa as a route for systemic drug delivery: a review. *Journal of pharmacy & pharmaceutical sciences : a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques*, 1, 15-30.
- SHUGAIV, E., TUZUN, E., MUTLU, M., KIYAT-ATAMER, A., KURTUNCU, M. & AKMAN-DEMIR, G. 2011. Mycophenolate mofetil as a novel immunosuppressant in the treatment of neuro-Behcet's disease with parenchymal involvement: presentation of four cases. *Clinical and experimental rheumatology*, 29, S64-7.
- SILVA, A. C., FARIA, M. R., FONTES, A., CAMPOS, M. S. & CAVALCANTI, B. N. 2009. Interleukin-1 beta and interleukin-8 in healthy and inflamed dental pulps. *J Appl Oral Sci*, 17, 527-32.
- SILVA, M. T. 2010. Secondary necrosis: the natural outcome of the complete apoptotic program. *FEBS letters*, 584, 4491-9.
- SISTIG, S., CEKIC-ARAMBASIN, A., RABATIC, S., VUCICEVIC-BORAS, V., KLEINHEINZ, J. & PIFFKO, J. 2001. Natural immunity in recurrent aphthous

- ulceration. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 30, 275-80.
- SKRZECZYNSKA-MONCZNIK, J., WLODARCZYK, A., ZABIEGLO, K., KAPINSKA-MROWIECKA, M., MAREWICZ, E., DUBIN, A., POTEMPA, J. & CICHY, J. 2012. Secretory leukocyte proteinase inhibitor-competent DNA deposits are potent stimulators of plasmacytoid dendritic cells: implication for psoriasis. *Journal of immunology*, 189, 1611-7.
- SMEDLY, L. A., TONNESEN, M. G., SANDHAUS, R. A., HASLETT, C., GUTHRIE, L. A., JOHNSTON, R. B., JR., HENSON, P. M. & WORTHEN, G. S. 1986. Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase. *The Journal of clinical investigation*, 77, 1233-43.
- SMITH, C. W., HOLLERS, J. C., PATRICK, R. A. & HASSETT, C. 1979. Motility and adhesiveness in human neutrophils. Effects of chemotactic factors. *The Journal of clinical investigation*, 63, 221-9.
- SONIS, S. T. 2004. The pathobiology of mucositis. *Nat Rev Cancer*, 4, 277-84.
- SPIRA, A., BEANE, J., SCHEMBRI, F., LIU, G., DING, C., GILMAN, S., YANG, X., CANTOR, C. & BRODY, J. S. 2004. Noninvasive method for obtaining RNA from buccal mucosa epithelial cells for gene expression profiling. *BioTechniques*, 36, 484-7.
- SPIVACK, S. D., HURTEAU, G. J., JAIN, R., KUMAR, S. V., ALDOUS, K. M., GIERTHY, J. F. & KAMINSKY, L. S. 2004. Gene-environment interaction signatures by quantitative mRNA profiling in exfoliated buccal mucosal cells. *Cancer research*, 64, 6805-13.
- STEINBERG, B. E. & GRINSTEIN, S. 2007. Unconventional roles of the NADPH oxidase: signaling, ion homeostasis, and cell death. *Science's STKE : signal transduction knowledge environment*, 2007, pe11.
- STOCKLEY, R., DE SOYZA, A., GUNAWARDENA, K., PERRETT, J., FORSMAN-SEMB, K., ENTWISTLE, N. & SNELL, N. 2013. Phase II study of a neutrophil elastase inhibitor (AZD9668) in patients with bronchiectasis. *Respiratory medicine*, 107, 524-33.
- STOCKLEY, R. A. 2002. Neutrophils and the pathogenesis of COPD. *Chest*, 121, 151S-155S.
- STOLLER, J. K. & ABOUSSOUAN, L. S. 2005. Alpha1-antitrypsin deficiency. *Lancet*, 365, 2225-36.
- STUDD, M., MCCANCE, D. J. & LEHNER, T. 1991. Detection of HSV-1 DNA in patients with Behcet's syndrome and in patients with recurrent oral ulcers by the polymerase chain reaction. *J Med Microbiol*, 34, 39-43.
- SULLIVAN, A. L., DAFFORN, T., HIEMSTRA, P. S. & STOCKLEY, R. A. 2008. Neutrophil elastase reduces secretion of secretory leukoprotease inhibitor (SLPI) by lung epithelial cells: role of charge of the proteinase-inhibitor complex. *Respir Res*, 9, 60.
- SUMI, Y., MURAMATSU, H., HATA, K., UEDA, M. & MURAMATSU, T. 2000. Secretory leukocyte protease inhibitor is a novel inhibitor of fibroblast-mediated collagen gel contraction. *Experimental cell research*, 256, 203-12.
- SUN, A., CHU, C. T., LIU, B. Y., WANG, J. T., LEU, J. S. & CHIANG, C. P. 2000. Expression of interleukin-2 receptor by activated peripheral blood lymphocytes upregulated by the plasma level of interleukin-2 in patients with recurrent aphthous ulcers. *Proceedings of the National Science Council, Republic of China. Part B, Life sciences*, 24, 116-22.

- SUN, Z. & YANG, P. 2004. Role of imbalance between neutrophil elastase and alpha 1-antitrypsin in cancer development and progression. *The lancet oncology*, 5, 182-90.
- SUZUKI, T., MORAES, T. J., VACHON, E., GINZBERG, H. H., HUANG, T. T., MATTHAY, M. A., HOLLENBERG, M. D., MARSHALL, J., MCCULLOCH, C. A., ABREU, M. T., CHOW, C. W. & DOWNEY, G. P. 2005. Proteinase-activated receptor-1 mediates elastase-induced apoptosis of human lung epithelial cells. *American journal of respiratory cell and molecular biology*, 33, 231-47.
- SZPADERSKA, A. M., ZUCKERMAN, J. D. & DIPIETRO, L. A. 2003. Differential injury responses in oral mucosal and cutaneous wounds. *Journal of dental research*, 82, 621-6.
- TABORDA, N., ZAPATA-BUILES, W., MONTOYA, C. & RUGELES, M. T. 2012. Short communication: Increased expression of secretory leukocyte protease inhibitor in oral mucosa of Colombian HIV type 1-exposed seronegative individuals. *AIDS Res Hum Retroviruses*, 28, 1059-62.
- TAGGART, C. C., LOWE, G. J., GREENE, C. M., MULGREW, A. T., O'NEILL, S. J., LEVINE, R. L. & MCELVANEY, N. G. 2001. Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. *The Journal of biological chemistry*, 276, 33345-52.
- TAKAHASHI, H., NUKIWA, T., YOSHIMURA, K., QUICK, C. D., STATES, D. J., HOLMES, M. D., WHANG-PENG, J., KNUTSEN, T. & CRYSTAL, R. G. 1988. Structure of the human neutrophil elastase gene. *The Journal of biological chemistry*, 263, 14739-47.
- TAKEI, H., ARAKI, A., WATANABE, H., ICHINOSE, A. & SENDO, F. 1996. Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis. *Journal of leukocyte biology*, 59, 229-40.
- TALACKO, A. A., GORDON, A. K. & ALDRED, M. J. 2010. The patient with recurrent oral ulceration. *Australian dental journal*, 55 Suppl 1, 14-22.
- TAYLOR, L. J., BAGG, J., WALKER, D. M. & PETERS, T. J. 1992. Increased production of tumour necrosis factor by peripheral blood leukocytes in patients with recurrent oral aphthous ulceration. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 21, 21-5.
- THOMPSON, R. C. & OHLSSON, K. 1986. Isolation, Properties, and Complete Amino-Acid-Sequence of Human Secretory Leukocyte Protease Inhibitor, a Potent Inhibitor of Leukocyte Elastase. *Proceedings of the National Academy of Sciences of the United States of America*, 83, 6692-6696.
- TISCHNER, D., WOESS, C., OTTINA, E. & VILLUNGER, A. 2010. Bcl-2-regulated cell death signalling in the prevention of autoimmunity. *Cell death & disease*, 1, e48.
- TOMEI, J. F., HIEMSTRA, P. S., HEINZEL-WIELAND, R. & KAUFFMAN, H. F. 1997. Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi. *The Journal of infectious diseases*, 176, 740-7.
- TOMEI, J. F., KOETER, G. H., HIEMSTRA, P. S. & KAUFFMAN, H. F. 1998. Secretory leukoprotease inhibitor: a native antimicrobial protein presenting a new therapeutic option? *Thorax*, 53, 114-6.
- TOYOSHIMA, T., KOCH, F., KAEMMERER, P., VAIRAKTARIS, E., AL-NAWAS, B. & WAGNER, W. 2009a. Expression of cytokeratin 17 mRNA in oral squamous cell carcinoma cells obtained by brush biopsy: preliminary results. *J Oral Pathol Med*, 38, 530-4.

- TOYOSHIMA, T., KOCH, F., KAEMMERER, P., VAIRAKTARIS, E., AL-NAWAS, B. & WAGNER, W. 2009b. Expression of cytokeratin 17 mRNA in oral squamous cell carcinoma cells obtained by brush biopsy: preliminary results. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 38, 530-4.
- TRACEY, K. J., FONG, Y., HESSE, D. G., MANOGUE, K. R., LEE, A. T., KUO, G. C., LOWRY, S. F. & CERAMI, A. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature*, 330, 662-4.
- TRAVIS, J. 1988. Structure, function, and control of neutrophil proteinases. *Am J Med*, 84, 37-42.
- TSAI, C., HATA, K., TORII, S., MATSUYAMA, M. & UEDA, M. 1995. Contraction potency of hypertrophic scar-derived fibroblasts in a connective tissue model: in vitro analysis of wound contraction. *Annals of plastic surgery*, 35, 638-46.
- TSOKOS, G. C. 2011. Systemic lupus erythematosus. *The New England journal of medicine*, 365, 2110-21.
- TSOUMAKIDOU, M., BOULOUKAKI, I., THIMAKI, K., TZANAKIS, N. & SIAFAKAS, N. M. 2010. Innate immunity proteins in chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. *Exp Lung Res*, 36, 373-80.
- TURAN, B., GALLATI, H., ERDI, H., GURLER, A., MICHEL, B. A. & VILLIGER, P. M. 1997. Systemic levels of the T cell regulatory cytokines IL-10 and IL-12 in Bechcet's disease; soluble TNFR-75 as a biological marker of disease activity. *The Journal of rheumatology*, 24, 128-32.
- UETA, E., OSAKI, T., YONEDA, K. & YAMAMOTO, T. 1993. Functions of salivary polymorphonuclear leukocytes (SPMNs) and peripheral blood polymorphonuclear leukocytes (PPMNs) from healthy individuals and oral cancer patients. *Clinical immunology and immunopathology*, 66, 272-8.
- URBAN, C. F., ERMERT, D., SCHMID, M., ABU-ABED, U., GOOSMANN, C., NACKEN, W., BRINKMANN, V., JUNGBLUT, P. R. & ZYCHLINSKY, A. 2009. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS pathogens*, 5, e1000639.
- VAN 'T WOUT, E. F., VAN SCHADEWIJK, A., SAVAGE, N. D., STOLK, J. & HIEMSTRA, P. S. 2012. alpha1-antitrypsin production by proinflammatory and antiinflammatory macrophages and dendritic cells. *American journal of respiratory cell and molecular biology*, 46, 607-13.
- VAN WETERING, S., VAN DER LINDEN, A. C., VAN STERKENBURG, M. A., RABE, K. F., SCHALKWIJK, J. & HIEMSTRA, P. S. 2000. Regulation of secretory leukocyte proteinase inhibitor (SLPI) production by human bronchial epithelial cells: increase of cell-associated SLPI by neutrophil elastase. *J Investig Med*, 48, 359-66.
- VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE PAEPE, A. & SPELEMAN, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology*, 3, RESEARCH0034.
- VENEMBRE, P., BOUTTEN, A., SETA, N., DEHOUX, M. S., CRESTANI, B., AUBIER, M. & DURAND, G. 1994. Secretion of alpha 1-antitrypsin by alveolar epithelial cells. *FEBS letters*, 346, 171-4.
- VERITY, D. H., WALLACE, G. R., VAUGHAN, R. W. & STANFORD, M. R. 2003. Behcet's disease: from Hippocrates to the third millennium. *Br J Ophthalmol*, 87, 1175-83.
- VIGNOLA, A. M., BONANNO, A., MIRABELLA, A., RICCOBONO, L., MIRABELLA, F., PROFITA, M., BELLIA, V., BOUSQUET, J. &

- BONSIGNORE, G. 1998. Increased levels of elastase and alpha1-antitrypsin in sputum of asthmatic patients. *American journal of respiratory and critical care medicine*, 157, 505-11.
- VILLANUEVA, E., YALAVARTHI, S., BERTHIER, C. C., HODGIN, J. B., KHANDPUR, R., LIN, A. M., RUBIN, C. J., ZHAO, W., OLSEN, S. H., KLINKER, M., SHEALY, D., DENNY, M. F., PLUMAS, J., CHAPEROT, L., KRETZLER, M., BRUCE, A. T. & KAPLAN, M. J. 2011. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *Journal of immunology*, 187, 538-52.
- VINCENT, S. D. & LILLY, G. E. 1992. Clinical, historic, and therapeutic features of aphthous stomatitis. Literature review and open clinical trial employing steroids. *Oral surgery, oral medicine, and oral pathology*, 74, 79-86.
- VISSERS, M. C., GEORGE, P. M., BATHURST, I. C., BRENNAN, S. O. & WINTERBOURN, C. C. 1988. Cleavage and inactivation of alpha 1-antitrypsin by metalloproteinases released from neutrophils. *The Journal of clinical investigation*, 82, 706-11.
- VITKOV, L., KLAPPACHER, M., HANNIG, M. & KRAUTGARTNER, W. D. 2009. Extracellular neutrophil traps in periodontitis. *Journal of periodontal research*, 44, 664-72.
- VOGELMEIER, C., AQUINO, T. O., O'BRIEN, C. D., PERRETT, J. & GUNAWARDENA, K. A. 2012. A Randomised, Placebo-Controlled, Dose-Finding Study Of AZD9668, An Oral Inhibitor of Neutrophil Elastase, in Patients with Chronic Obstructive Pulmonary Disease Treated with Tiotropium. *COPD*.
- VOGELMEIER, C., GILLISSEN, A. & BUHL, R. 1996. Use of secretory leukoprotease inhibitor to augment lung antineutrophil elastase activity. *Chest*, 110, 261S-266S.
- VON KOCKRITZ-BLICKWEDE, M., GOLDMANN, O., THULIN, P., HEINEMANN, K., NORRBY-TEGLUND, A., ROHDE, M. & MEDINA, E. 2008. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood*, 111, 3070-80.
- WAHL, S. M., MCNEELY, T. B., JANOFF, E. N., SHUGARS, D., WORLEY, P., TUCKER, C. & ORENSTEIN, J. M. 1997a. Secretory leukocyte protease inhibitor (SLPI) in mucosal fluids inhibits HIV-I. *Oral Dis*, 3 Suppl 1, S64-9.
- WAHL, S. M., WORLEY, P., JIN, W., MCNEELY, T. B., EISENBERG, S., FASCHING, C., ORENSTEIN, J. M. & JANOFF, E. N. 1997b. Anatomic dissociation between HIV-1 and its endogenous inhibitor in mucosal tissues. *The American journal of pathology*, 150, 1275-84.
- WALKER, D. M. 2004. Oral mucosal immunology: an overview. *Annals of the Academy of Medicine, Singapore*, 33, 27-30.
- WANG, S. L., MILLES, M., WU-WANG, C. Y., LIU, J., SLOMIANY, A. & SLOMIANY, B. L. 1990. Identification of epidermal growth factor receptor in human buccal mucosa. *Archives of oral biology*, 35, 823-8.
- WAUGH, A., GRANT, A., CHAMBERS, G., ROSS, J. S. A., PHYSIOLOGY IN, H., ILLNESS & WILSON, K. J. W. A. 2006. *Ross and Wilson anatomy and physiology in health and illness*, Edinburgh, Churchill Livingstone.
- WEBB, P. R., WANG, K. Q., SCHEEL-TOELLNER, D., PONGRACZ, J., SALMON, M. & LORD, J. M. 2000. Regulation of neutrophil apoptosis: a role for protein kinase C and phosphatidylinositol-3-kinase. *Apoptosis : an international journal on programmed cell death*, 5, 451-8.
- WEBSTER, R. O., WYSOLMERSKI, R. B. & LAGUNOFF, D. 1986. Enhancement of human polymorphonuclear leukocyte adherence to plastic and endothelium by phorbol myristate acetate. Comparison with human C5a. *The American journal of pathology*, 125, 369-78.

- WELDON, S., MCNALLY, P., MCELVANEY, N. G., ELBORN, J. S., MCAULEY, D. F., WARTELE, J., BELAAOUAJ, A., LEVINE, R. L. & TAGGART, C. C. 2009. Decreased levels of secretory leucoprotease inhibitor in the Pseudomonas-infected cystic fibrosis lung are due to neutrophil elastase degradation. *Journal of immunology*, 183, 8148-56.
- WEN, J., NIKITAKIS, N. G., CHAISUPARAT, R., GREENWELL-WILD, T., GLIOZZI, M., JIN, W., ADLI, A., MOUTSOPOULOS, N., WU, T., Warburton, G. & WAHL, S. M. 2011. Secretory leukocyte protease inhibitor (SLPI) expression and tumor invasion in oral squamous cell carcinoma. *The American journal of pathology*, 178, 2866-78.
- WESTIN, U., FRYKSMARK, U., POLLING, A. & OHLSSON, K. 1994. Localisation of secretory leukocyte proteinase inhibitor mRNA in nasal mucosa. *Acta otolaryngologica*, 114, 199-202.
- WESTIN, U., LUNDBERG, E., WIHL, J. A. & OHLSSON, K. 1999a. The effect of immediate-hypersensitivity reactions on the level of SLPI, granulocyte elastase, alpha1-antitrypsin, and albumin in nasal secretions, by the method of unilateral antigen challenge. *Allergy*, 54, 857-64.
- WESTIN, U., POLLING, A., LJUNGKRANTZ, I. & OHLSSON, K. 1999b. Identification of SLPI (secretory leukocyte protease inhibitor) in human mast cells using immunohistochemistry and in situ hybridisation. *Biological chemistry*, 380, 489-93.
- WITKO-SARSAT, V., RIEU, P., DESCAMPS-LATSCHA, B., LESAVRE, P. & HALBWACHS-MECARELLI, L. 2000. Neutrophils: molecules, functions and pathophysiological aspects. *Laboratory investigation; a journal of technical methods and pathology*, 80, 617-53.
- WRIGHT, H. L., MOOTS, R. J., BUCKNALL, R. C. & EDWARDS, S. W. 2010. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology*, 49, 1618-31.
- YAGER, D. R. & NWOMEH, B. C. 1999. The proteolytic environment of chronic wounds. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society*, 7, 433-41.
- YAMAKAWA, Y., SUGITA, Y., NAGATANI, T., TAKAHASHI, S., YAMAKAWA, T., TANAKA, S., NAKAMURA, S., OHNO, S., SEKIHARA, H., OKUDA, K. & NAKAJIMA, H. 1996. Interleukin-6 (IL-6) in patients with Behcet's disease. *Journal of dermatological science*, 11, 189-95.
- YAMAMOTO, T., OSAKI, T., YONEDA, K. & UETA, E. 1994. Cytokine production by keratinocytes and mononuclear infiltrates in oral lichen planus. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 23, 309-15.
- YAMASHITA, N., KANEOKA, H., KANEKO, S., TAKENO, M., ONEDA, K., KOIZUMI, H., KOGURE, M., INABA, G. & SAKANE, T. 1997. Role of gammadelta T lymphocytes in the development of Behcet's disease. *Clinical and experimental immunology*, 107, 241-7.
- YAZICI, H., PAZARLI, H., BARNES, C. G., TUZUN, Y., OZYAZGAN, Y., SILMAN, A., SERDAROGLU, S., OGUZ, V., YURDAKUL, S., LOVATT, G. E. & ET AL. 1990. A controlled trial of azathioprine in Behcet's syndrome. *The New England journal of medicine*, 322, 281-5.
- YOKOTA, K., HAYASHI, S., FUJII, N., YOSHIKAWA, K., KOTAKE, S., ISOGAI, E., OHNO, S., ARAKI, Y. & OGUMA, K. 1992. Antibody response to oral streptococci in Behcet's disease. *Microbiol Immunol*, 36, 815-22.
- YOSIPOVITCH, G., SHOHAT, B., BSHARA, J., WYSENBECK, A. & WEINBERGER, A. 1995. Elevated serum interleukin 1 receptors and interleukin 1B in patients

- with Behcet's disease: correlations with disease activity and severity. *Israel journal of medical sciences*, 31, 345-8.
- YOUSEFI, S., GOLD, J. A., ANDINA, N., LEE, J. J., KELLY, A. M., KOZLOWSKI, E., SCHMID, I., STRAUMANN, A., REICHENBACH, J., GLEICH, G. J. & SIMON, H. U. 2008. Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nature medicine*, 14, 949-53.
- ZABEL, P., SCHADE, F. U. & SCHLAAK, M. 1993. Inhibition of endogenous TNF formation by pentoxifylline. *Immunobiology*, 187, 447-63.
- ZANI, M. L., BARANGER, K., GUYOT, N., DALLET-CHOISY, S. & MOREAU, T. 2009. Protease inhibitors derived from elafin and SLPI and engineered to have enhanced specificity towards neutrophil serine proteases. *Protein science : a publication of the Protein Society*, 18, 579-94.
- ZHOU, Z. Y., CHEN, S. L., SHEN, N. & LU, Y. 2012. Cytokines and Behcet's disease. *Autoimmunity reviews*, 11, 699-704.
- ZOUBOULIS, C. C., KATSANTONIS, J., KETTELER, R., TREUDLER, R., KAKLAMANI, E., HORNEMANN, S., KAKLAMANIS, P. & ORFANOS, C. E. 2000. Adamantiades-Behcet's disease: interleukin-8 is increased in serum of patients with active oral and neurological manifestations and is secreted by small vessel endothelial cells. *Archives of dermatological research*, 292, 279-84.

INVITATION TO PARTICIPATE (Healthy Volunteers)

IMMUNOREGULATION AT THE MUCOSAL BARRIER

Bart's and The London Queen Mary's School of Medicine and Dentistry, University of
London

Version 1: February 2004

We invite you to take part in a research study which we think may be important. The information which follows tells you about it. It is important that you understand what is in this leaflet. It says what will happen if you take part and what the risks might be. Try to make sure you know what will happen to you if you decide to take part. Whether or not you do take part is entirely your choice. Please ask any questions you want to about the research and we will try our best to answer them.

You have been invited to participate in this study because you are a healthy individual. We wish to collect some tissue from your mouth (biopsy), saliva, urine and blood samples.

This is because we think that inflammation in the mouth may have a more widespread effect in determining whether patients develop other features of their particular illness. Mouth inflammation may predict severity or activation of systemic disease or disease at distant sites such as the eye.

Understanding the nature of this may allow development of preventative treatments or strategies to reduce systemic effects of oral inflammation in patients with oral mucosal disease. You are unlikely to directly benefit from this research yourself, but we hope that the results will benefit patients in the future.

We require healthy volunteers in order to find out whether the effects of inflammation on the mucosa of patients are different to the mucosa of healthy volunteers.

What would I do in the study if I took part?

If you agree to participate in this study you will initially be asked to complete a confidential health questionnaire; you will also undergo a physical examination, including blood pressure, pulse rate and heart rhythm. We will also require a routine blood sample from your arm (about an egg-cup full), a sample of spit (1/2 egg-cup) and a urine sample.

If you are female, we will ask your permission to perform a pregnancy test; as we would prefer you not to take part in this study if you are pregnant. None of the tests we intend to do will harm an unborn baby, but the skin heals less well in pregnancy and we would prefer to avoid surgery in pregnancy unless absolutely necessary. Please inform us if you think you may be pregnant.

There are several components to this study:

On entry to the study we will take a blood sample and saliva. The blood sample will be taken as part of your routine screen. We will take 50 ml (egg cup full) of blood, 25ml (1/2 egg cup of spit) and 10 ml of urine. Blood tests may cause a very small discomfort when the needle is put into the vein, but this is momentary. The 25 ml of saliva will be taken by asking you to spit into a small container over a period of not more than 5 mins. The urine sample will be collected in private. Another two blood tests requiring the same amount of blood will be taken over the period of study.

A small mouth biopsy will be taken from the inside of your cheek or lip. This will be 6mm (the size of the tip of your small finger). You will be given a pain killing injection which will numb the area to be biopsied. This is similar to having an injection at your dentist. This painkilling injection does sting, but only momentarily, and it is very effective and works quickly. You will not be able to feel any discomfort for the rest of the procedure. A stitch will be used to hold the mucosa together afterwards; this will need to be removed by us 10 days after the procedure. Each mouth sample will be frozen and stored prior to examination using molecular or cellular technology. You are very unlikely to develop any problems following the above tests.

Very occasionally, however, a mouth biopsy or a blood test can be associated with bruising, bleeding or infection.

If you are female we will ask your permission to perform a pregnancy test, as we would prefer you not to take part in this study if you are pregnant. None of these tests will harm an unborn baby, but the skin heals less well in pregnancy and we would prefer to avoid surgery in pregnancy unless absolutely necessary. Please inform us if you think you might be pregnant.

The above tests will involve you attending either the Royal London Hospital or St. Thomas' Hospital not both (this will depend on the site that you normally attend clinic). Tissue samples taken from you may be stored for a period of no more than 5 years, and will not be used for any other purposes other than this study.

Similarly, any blood samples taken from you will only be used for this study.

If you have any questions concerning this study please feel free to ask us. We can provide you with further information if you wish. Contact names and telephone numbers are given below.

You don't have to join the study. You are free to decide not to be in this trial or to drop out at any time. If you decide not to be in the study, or drop out, this will not put at risk your ordinary medical care.

What happens if you are worried or if there is an emergency? You will always be able to contact an investigator to discuss your concerns and/or to get help:

Name: Prof.F.Fortune

Address: Centre for Clinical and Diagnostic Oral Science, Clinical Sciences Building, 2 Newark Street, London E1 2AT

Telephone number: 020-7882 7169

What happens if something goes wrong?

We believe that this study is basically safe and do not expect you to suffer any harm or injury because of your participation in it. However, we carry insurance to make sure that if your health does suffer as a result of your being in the study then you will be compensated. In such a situation, you will not have to prove that the harm or injury which affects you is anyone's fault. If you are not happy with any proposed compensation, you may have to pursue your claim through legal action.

INVITATION TO PARTICIPATE (Patients)
Immunoregulation at the mucosal barrier

Version 1: February 2004

Bart's and The London Queen Mary's School of Medicine and Dentistry, University of London

We invite you to take part in a research study which we think may be important. The information which follows tells you about it. It is important that you understand what is in this leaflet. It says what will happen if you take part and what the risks might be. Try to make sure you know what will happen to you if you decide to take part. Whether or not you do take part is entirely your choice and any decision will not affect the treatment you receive. Please ask any questions you want to about the research and we will try our best to answer them.

Why have we approached you?

You have been invited to participate in this study because you have an inflammatory condition which may affect the mouth. We wish to collect some tissue from your mouth (biopsy), saliva, urine and blood samples.

This is because we think that inflammation in the mouth causing ulceration may have a more widespread effect in determining whether you may develop other features of your particular illness. Mouth ulceration may predict severity or activation of your disease. You are unlikely to benefit directly from this research yourself, but we hope that the results of these studies will benefit similar patients in the future.

What would I do in the study if I took part?

If you agree to participate in this study you will initially be asked to complete a confidential health questionnaire; you will also undergo a physical examination, including blood pressure, pulse rate and heart rhythm. We will also require a routine blood sample from your arm (about an egg-cup full, 50 ml), a samples of spit (1/2 egg cup, 25 ml) and a urine sample (10 ml). A drugs of abuse screen will also be performed.

If you are female, we will ask your permission to perform a pregnancy test; we would prefer that you do not to take part in this study if you are pregnant. None of the tests we intend to do will harm an unborn baby, but the mucosa (tissue) heals less well in pregnancy. We would prefer to avoid biopsies in pregnancy unless absolutely necessary. Please inform us if you think you may be pregnant.

There are several components to the study:

On entry to the study we will take a blood sample and saliva. The blood sample will be taken as part of your routine screen. We will take 50 ml (1 egg cup full) of blood, 25 ml (1/2 egg-cup) of spit and 10ml of urine. Blood tests may cause a very small discomfort when the needle is put into the vein, but this is momentary. The saliva will be collected by asking you to spit into a small container over a period of not more than 5 mins. You will collect the urine sample in private.

Another two blood tests requiring the same amount of blood will be taken over the period of study.

A small mouth biopsy (6 mm, about the size of the tip of your small finger.) will be taken from the inside of your cheek or lip in an area where there is an ulcer or the tissue is very inflamed. You will be given a pain killing injection which will numb the area to be biopsied. This is similar to having an injection at your dentist. This painkilling injection does sting, but only momentarily, and it is very effective and works quickly. You will not be able to feel any discomfort for the rest of the procedure. A stitch will be used to hold the mucosa together afterwards; this will need to be removed by us 10 days

after the procedure. Each mouth sample will be frozen and stored prior to examination using molecular or cellular technology. We would like to see what is happening in the inflamed and ulcerated areas.

You are very unlikely to develop any problems following the above tests. Very occasionally, however, a mucosal biopsy or a blood test can be associated with bruising, bleeding or infection.

The above tests will involve you attending either the Royal London Hospital or St. Thomas' Hospital depending on where you are registered as a patient.

If you have any questions concerning this study, please feel free to ask us. We can provide you with further information if you wish. Contact names and telephone numbers are given below.

Tissue samples taken from you may be stored for a period of no more than 5 years, and will not be used for any other purposes other than this study.

Similarly, any blood samples taken from you will only be used for this study.

You don't have to join the study. You are free to decide not to be in this trial or to drop out at any time. If you decide not to be in the study, or drop out, this will not put at risk your ordinary medical care.

What happens if you are worried or if there is an emergency? You will always be able to contact an investigator to discuss your concerns and/or to get help:

Name: Prof.F.Fortune

Address: Centre for Clinical and diagnostic Oral Science, Clinical Sciences Building, 2 Newark Street, London E1 2AT

Telephone number: 020-7882 7154/8

What happens if something goes wrong?

We believe that this study is basically safe and do not expect you to suffer any harm or injury because of your participation in it. However, Barts and The London NHS Trust has agreed that if your health does suffer as a result of your being in the study then you will be compensated. In such a situation, you will not have to prove that the harm or injury which affects you is anyone's fault. If you are not happy with any proposed compensation, you may have to pursue your claim through legal action.

WRITTEN CONSENT FORM:

Title of research proposal:

REC Number:

Name of Patient/Volunteer (Block Capitals):

Address:

(Delete if unnecessary to the research project)

- The study organisers have invited me to take part in this research. ☐
- I understand what is in the leaflet about the research. I have a copy of the leaflet to keep. ☐
- I have had the chance to talk and ask questions about the study. ☐
- I know what my part will be in the study and I know how long it will take. ☐
- I have been told about any special drugs, operations, tests or other checks I might be given. ☐
- I know how the study may affect me. I have been told if there are possible risks. ☐
- I understand that I should not actively take part in more than 1 research study at a time. ☐
- I know that the local North East London Health Authority Research Ethics Committee has seen and agreed to this study. ☐
- I understand that personal information is strictly confidential: I know the only people who may see information about my part in the study are the research team or an official representative of the organisation which funded the research. ☐
- I understand that my personal information may be stored on a computer. If this is done then it will not affect the confidentiality of this information. All such storage of information must comply with the 1998 Data Protection Act. ☐
- I know that the researchers will/might tell my general practitioner (GP) about my part in the study. ☐
- I freely consent to be a subject in the study. No-one has put pressure on me. ☐
- I know that I can stop taking part in the study at any time. ☐
- I know if I do not take part I will still be able to have my normal treatment. ☐
- As a medical/ nursing student or qualified staff, I understand that agreement or Refusal to take part will make no difference to the results of my course or my career. ☐
- I know that if there are any problems, I can contact:

Dr/Mr/Ms.....

Tel. No. Bleep No./Ext.

Patient's/Volunteer's: Signature

.....

Witness's signature

.....

BEHCET'S DISEASE CLINICAL INFORMATION FORM

Date of Sample

Name

Hospital Number

Demographics

Age

Behcet's Phenotype:

Active

Quiet

Mouth

Genital

Eyes

Joints

Skin: Folliculitis

Erythema nodosum

Vascular

CNS

BD Activity

Relapse

Quiet

Current Drugs

Other Remarks:

Behçet's Disease Clinical Activity Information Form

Date of sample		
Name		
Hospital Number		
Diagnosis:		
Behcet's Phenotype	Active: (tick if applies)	Quiet: (tick if applies)
Mouth		
Genital		
Eyes		
Joints		
Skin: Folliculitis		
Erythema nodosum		
Pustule		
Vascular		
CNS		
GI tract		
Fatigue		
BD Activity (circle one)	Relapse:	Quiet:
Date of last respiratory tract infection (cold/flu etc):		
Past Medical history:		
Current drugs and Dosage:		
Current smoker?	Y/N Cigs/day for years
Past smoker?	Y/N	Date of quitting:
Ulcer Severity Score:		
BDCAF score:		

Patient Details

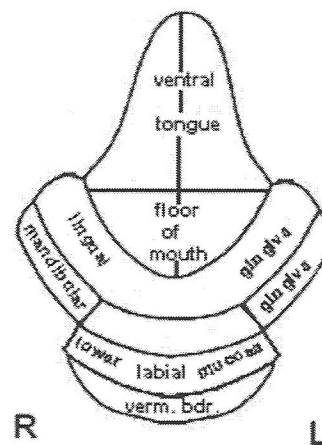
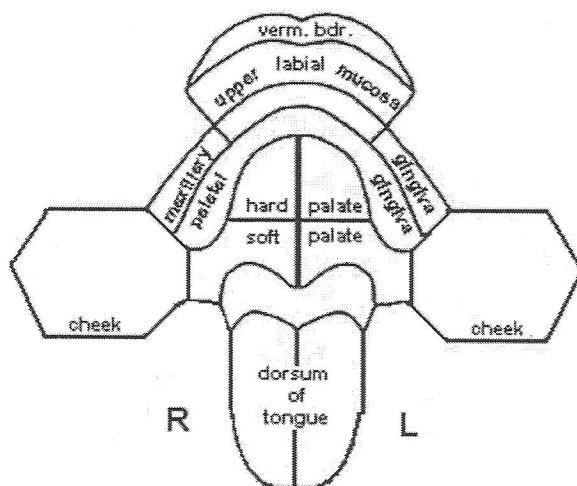
Sex: M F DOB: _____ / _____ / _____

BD Pemphigus PV CrD RAS Other_____

Length of time ulcer(s) have been present: _____ days/ weeks/ months

Smoking status: ☐ No ☐ Yes ☐ Occasional (frequency _____)

Site of oral ulcer(s) to be extracted:



Additional Notes:

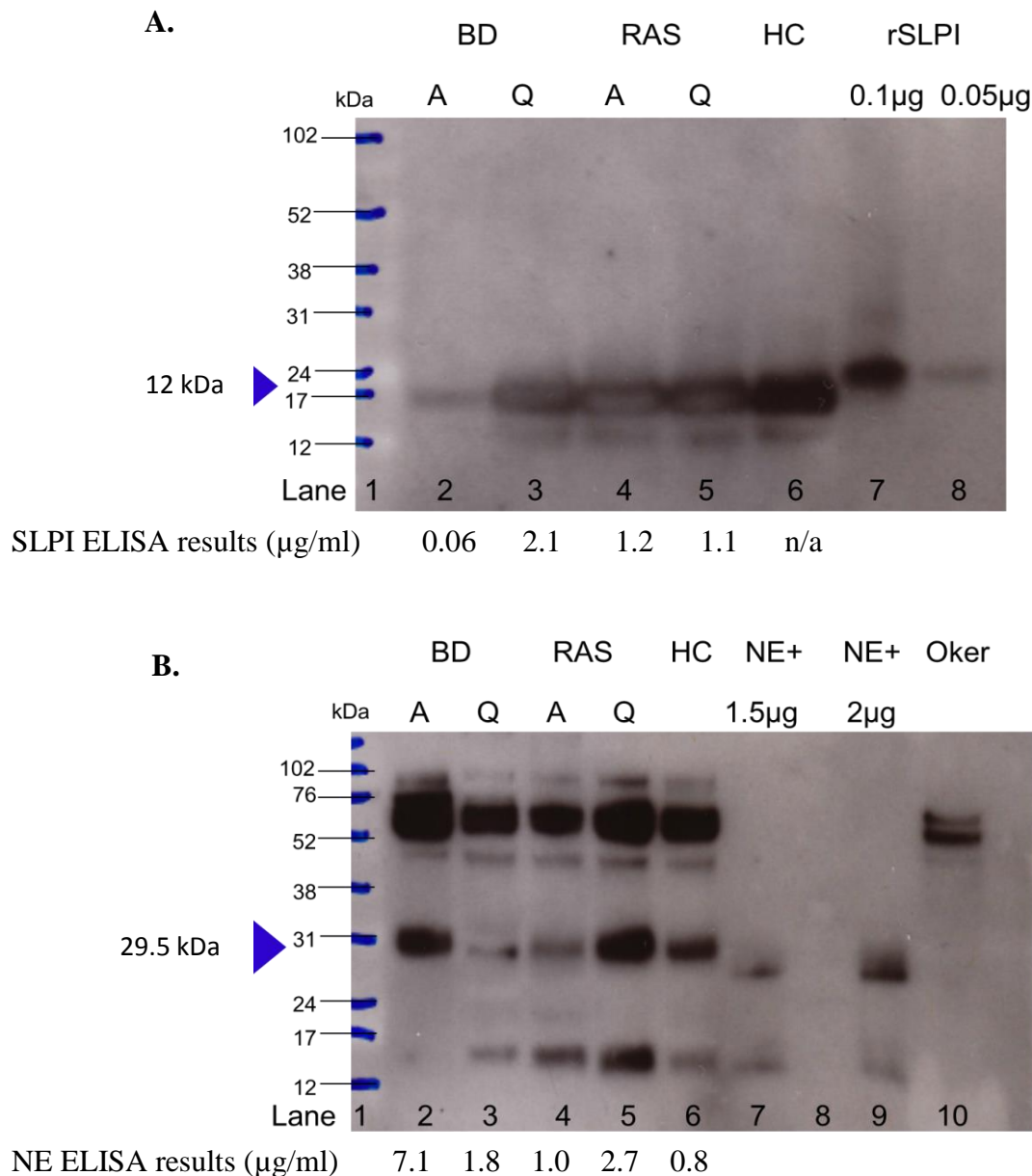


Figure 4.3.3.1. SLPI and NE in Saliva. The total protein was quantified and 10 µg loaded per well. **A.** Polyclonal SLPI antibody was diluted 1:500 and incubated for 1 hr at RT. After washing, a donkey, anti-goat secondary antibody was diluted to 1:3000 and incubated with the membrane for 1 hr at RT. Lane 1: Rainbow Full Range Protein Marker (GE Healthcare). Lane 2: BDa (active ulcer) saliva. Lane 3: BDq (quiet, no ulcer) saliva. Lane 4: RASa saliva. Lane 5: RASq saliva. Lane 6: HC saliva. Lane 7 and 8: recombinant SLPI (rSLPI, 12-14 kDa). **B.** Monoclonal NE-HRP antibody was diluted 1:500 and incubated for 1 hr at RT. Lane 7 and 9: purified human NE, 29.5 kDa, positive control (NE+), no DTT added or heat treatment. Lane 8: Empty. Lane 10: Oral keratinocyte (Oker) isolated culture supernatant as a NE negative control.

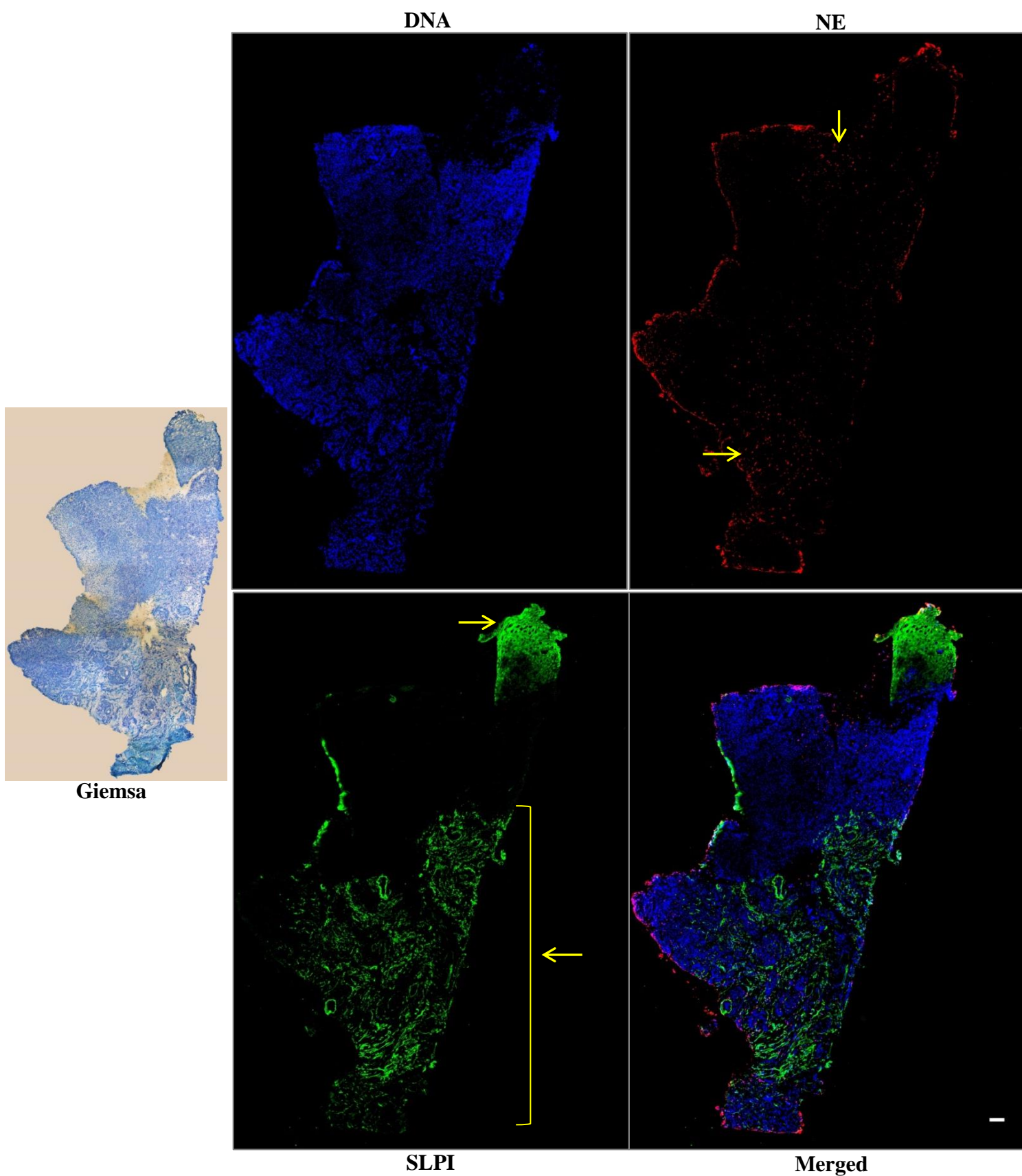


Fig. 4.3.6.1.1. Oral Ulcer Biopsy from Non-BD Patient. Bx1. Clinical investigation for hyperplasia & ulcerative changes. Identified as Bx1. DAPI DNA (blue), NE (red), SLPI (green). Arrows indicate main areas of positive staining for NE and SLPI. 10X magnification. Scale bar 100 μ m.

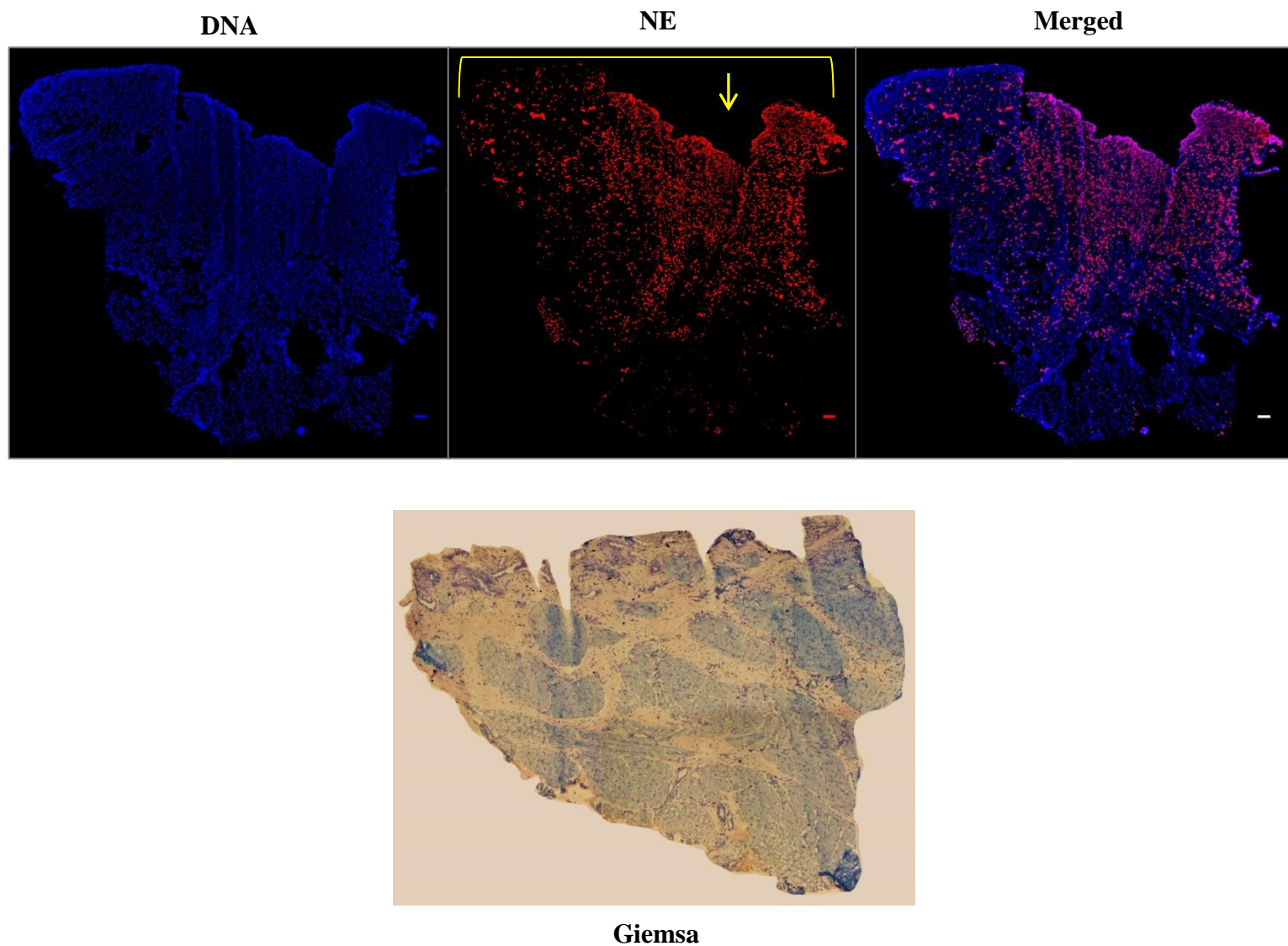


Fig. 4.3.6.1.2. Oral Ulcer Biopsy from BD Patient. Bx2. Minor and herpetiform ulcers for 10 days. DAPI DNA (blue), NE (red), SLPI (green). Arrows indicate main areas of positive staining for NE and SLPI. Giemsa-stained biopsy included for orientation. 10X magnification. Scale bar 100 μ m.

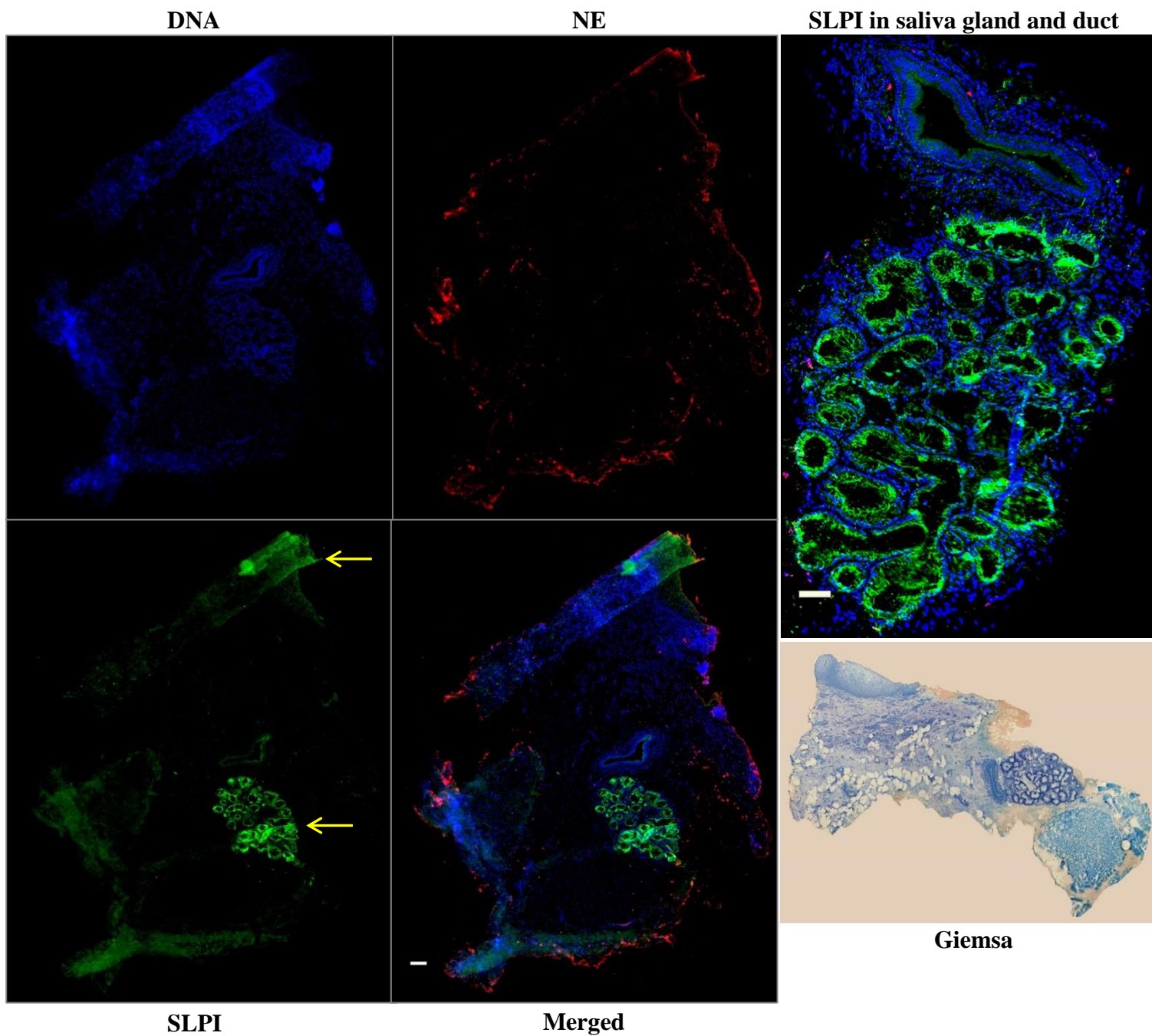


Fig. 4.3.6.1.3. Oral Ulcer Biopsy from BD Patient. Bx3. DAPI DNA (blue), NE (red), SLPI (green). Arrows indicate main areas of positive staining for SLPI. Enlarged image shows a minor saliva gland and duct from the buccal mucosa which stained positive for SLPI. Giemsa-stained biopsy was included for orientation. 10X magnification. Scale bar 100 μ m.

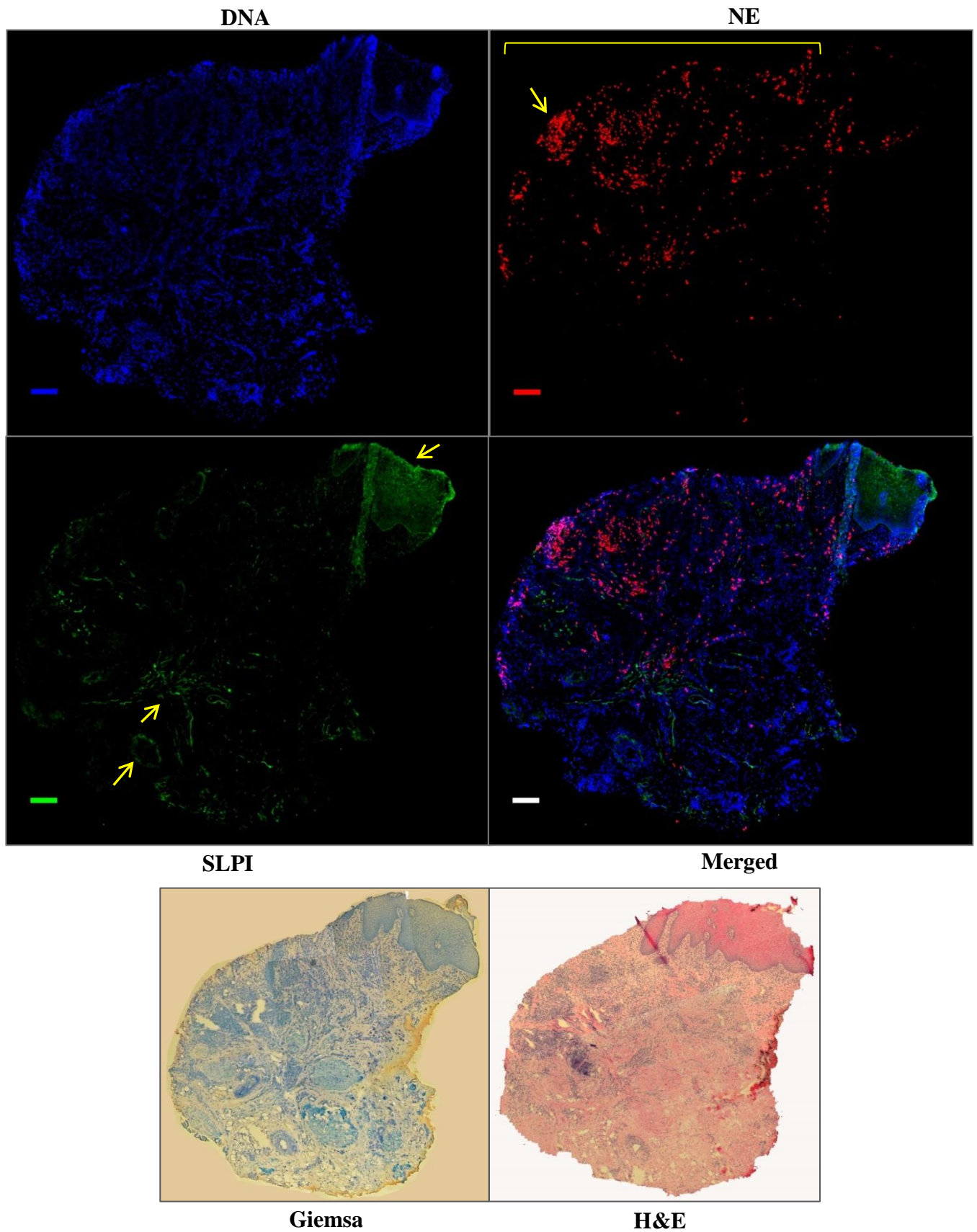


Fig. 4.3.6.1.4. Oral Ulcer Biopsy from BD Patient. Bx4. Patient had a major ulcer for 6 days prior to excision. DAPI DNA (blue), NE (red), SLPI (green). Arrows indicate main areas of positive staining. Giemsa and H&E stained biopsy sections were included for orientation. 10X magnification. Scale bar 100 μ m.

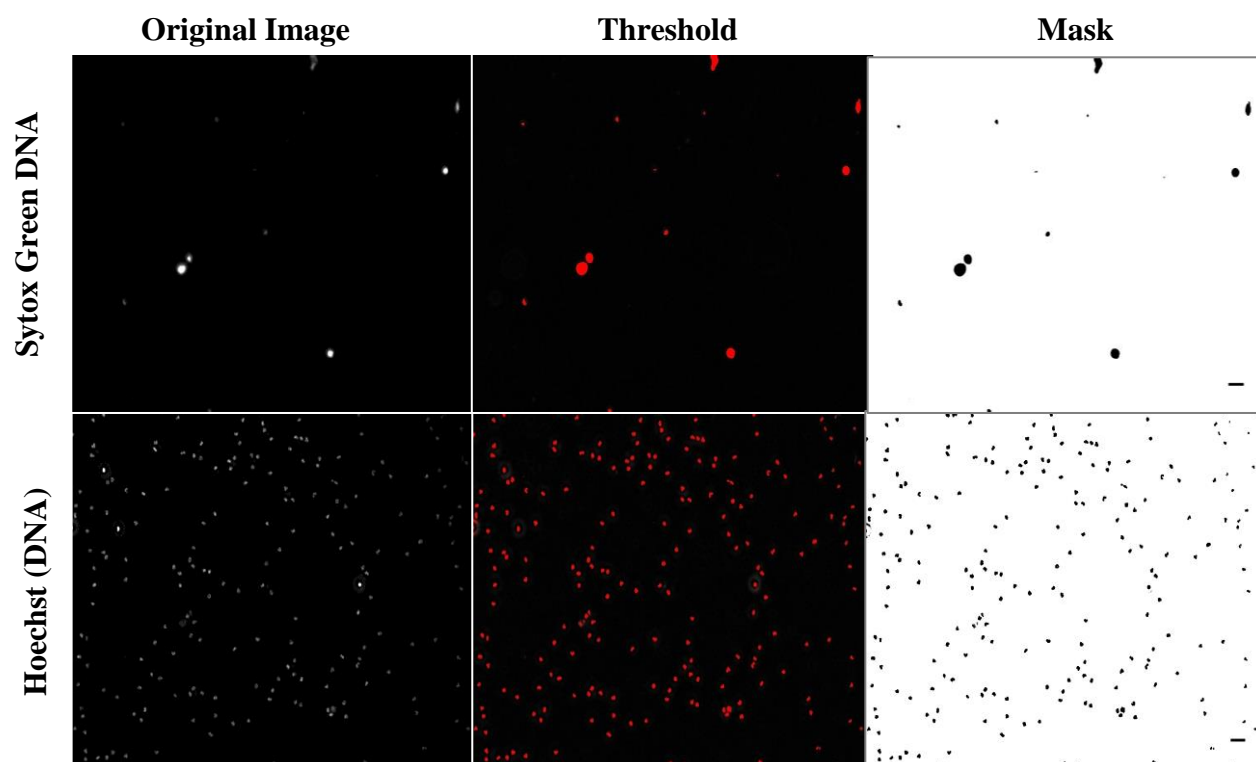


Fig 5.2.3.2.1.1. Simultaneous Images in GFP and DAPI Channels. Sytox[®] green attaches exclusively to extracellular DNA while Hoechst 33342 is able to penetrate the nuclear membrane and stain intracellular, intact DNA. Using FIJI/ImageJ software, minimum thresholds would be automatically set to recognise positively stained cells in each channel. Triplicate images were tallied to determine the mean percentage of sytox⁺ cells for each culture treatment. X20 magnification using air objective on Timelapse microscope. Scale bar 25 μ m.

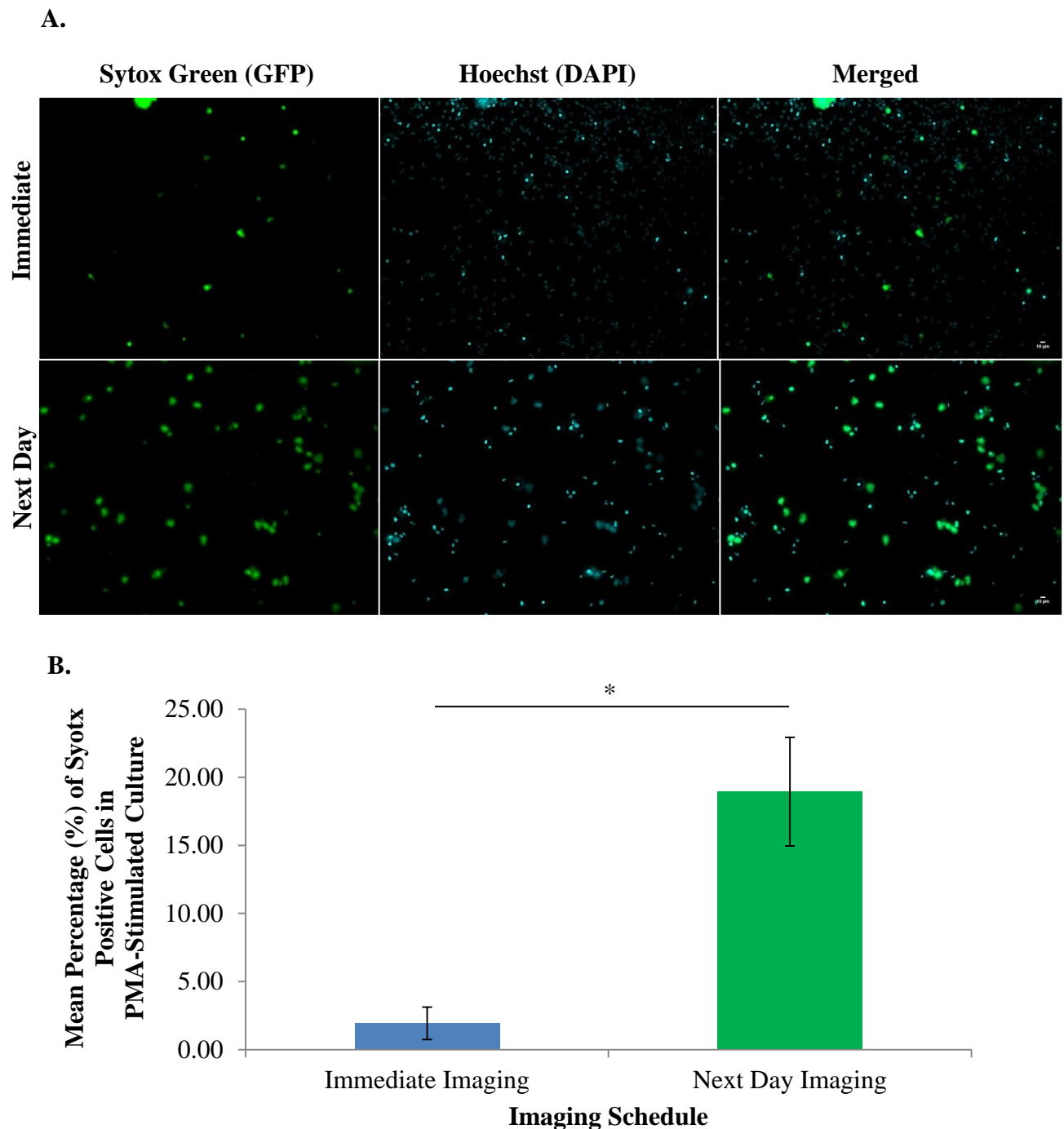


Fig. 5.3.1.1.1. A. and B. Representative Results of Increased Sytox⁺ Neutrophils During Next Day Imaging of the Same 1 hr PMA-Stimulated HC Neutrophil Culture. **A.** Top images are of the culture 1 hr after cells were stimulated with 100 nM PMA. Images were taken within 1 hr of adding sytox[®] green and Hoechst DNA stains to the culture media with live neutrophils. Bottom images were taken from the same HC PMA-stimulated culture within 24 hrs. **B. The Mean Difference Between Sytox⁺ HC Neutrophils when Imaging the Same Culture at Different Times.** Error bars represent \pm SEM of the mean percentage of sytox positive cells ($\geq 100 \mu\text{m}^2$) from multiple images of biological replicates. Mann Whitney U, 2-tailed, non-parametric test, * $p < 0.05$.

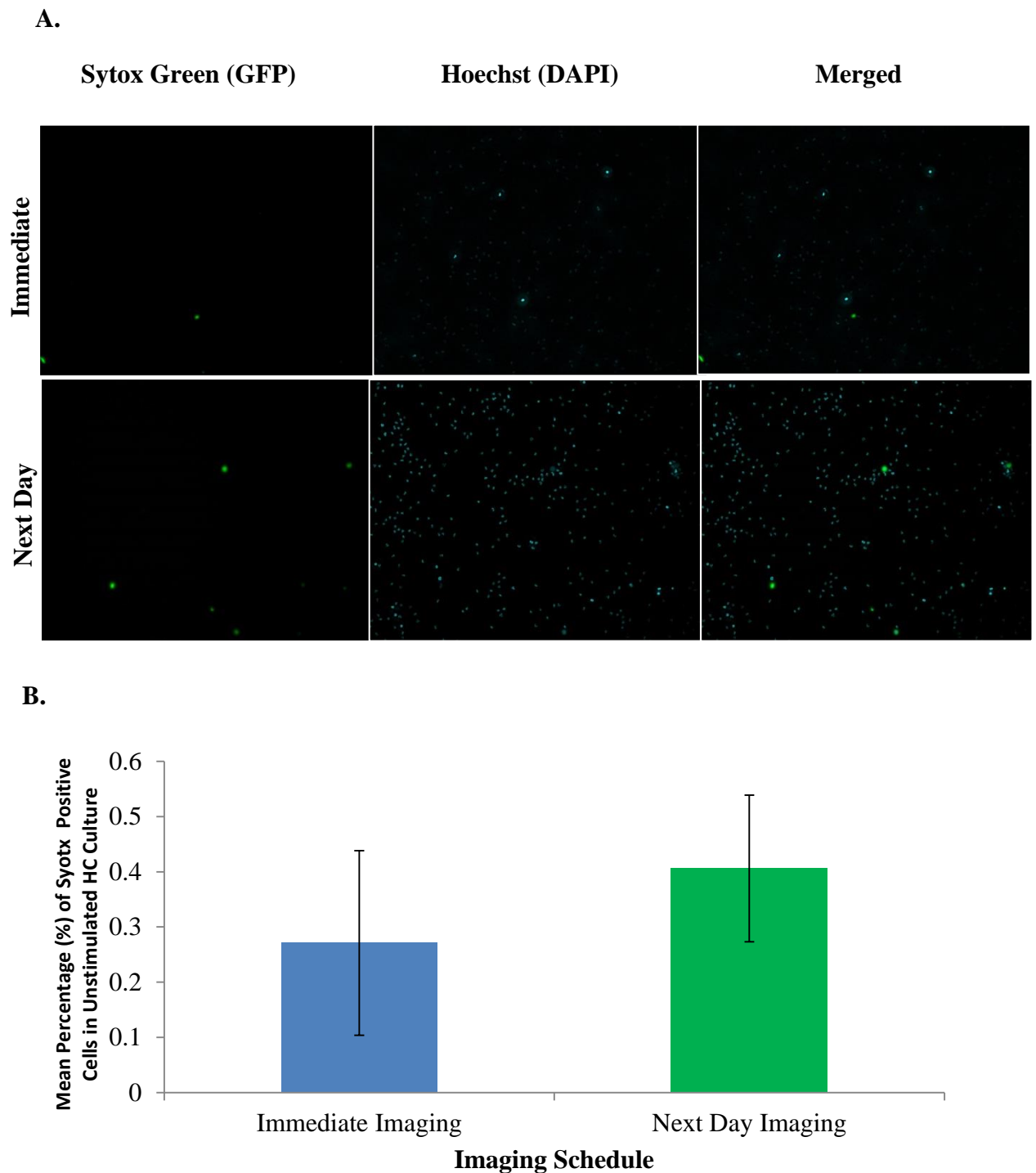


Fig. 5.3.1.1.2, A. and B. An Example of Increased Sytox⁺ Neutrophils During Next Day Imaging of the Same Unstimulated HC Neutrophil Culture. A. Top images were taken within 1 hr of adding Sytox green and Hoechst stains to the culture media with live neutrophils. Bottom images were taken from the same HC culture within 24 hours. **B. The Mean Difference Between Sytox⁺ HC Neutrophils when Imaging the Same Culture at Different Times.** Error bars represent \pm SEM of mean percentage of sytox positive cells ($\geq 100 \mu\text{m}^2$) from multiple images of biological replicates.

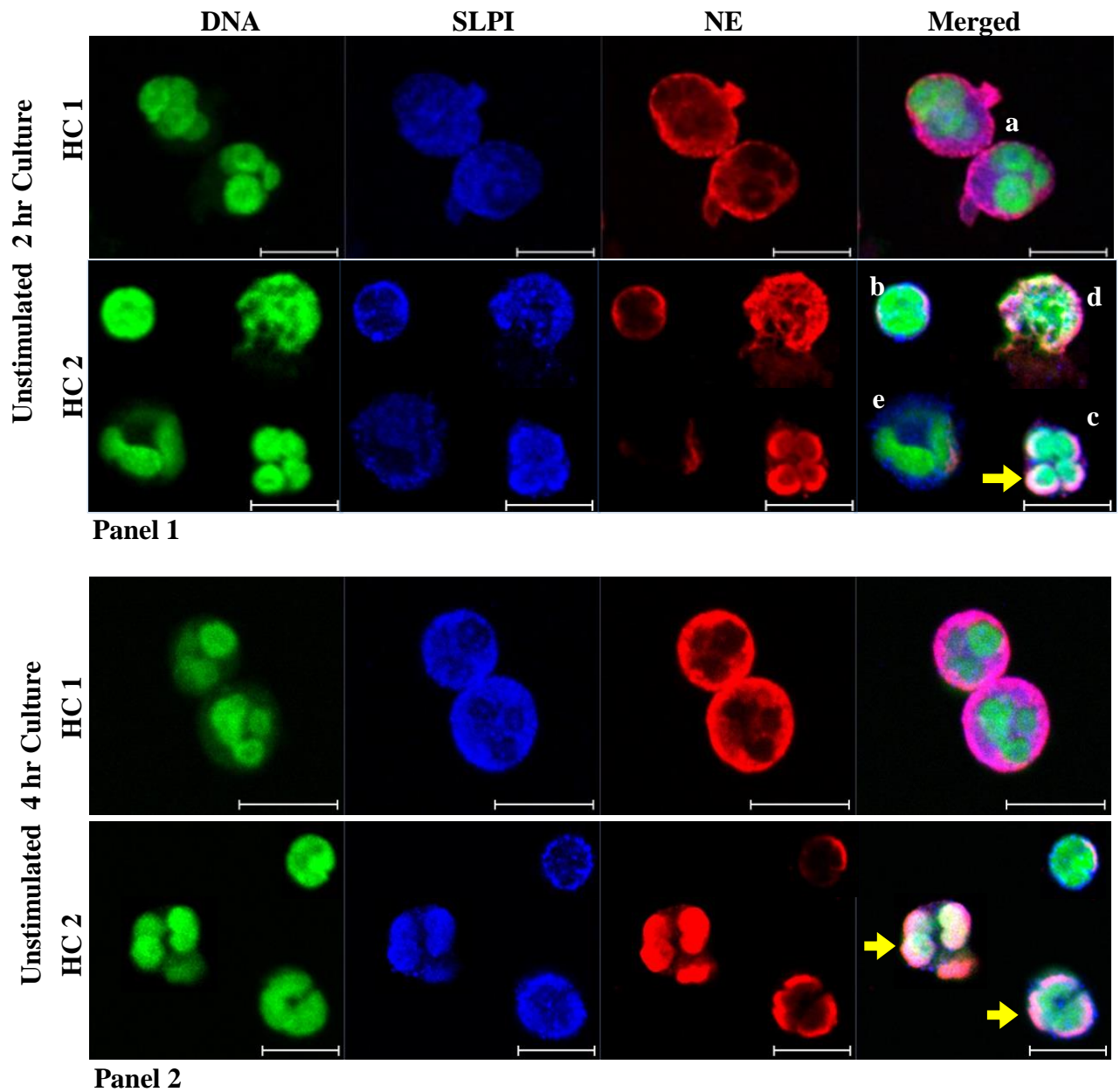


Fig. 5.3.2.1.2.1.1. Unstimulated HC Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in Unstimulated Cultures. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs (Panel 1) and 4 hrs (Panel 2) in culture media without NE inhibitor before being fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

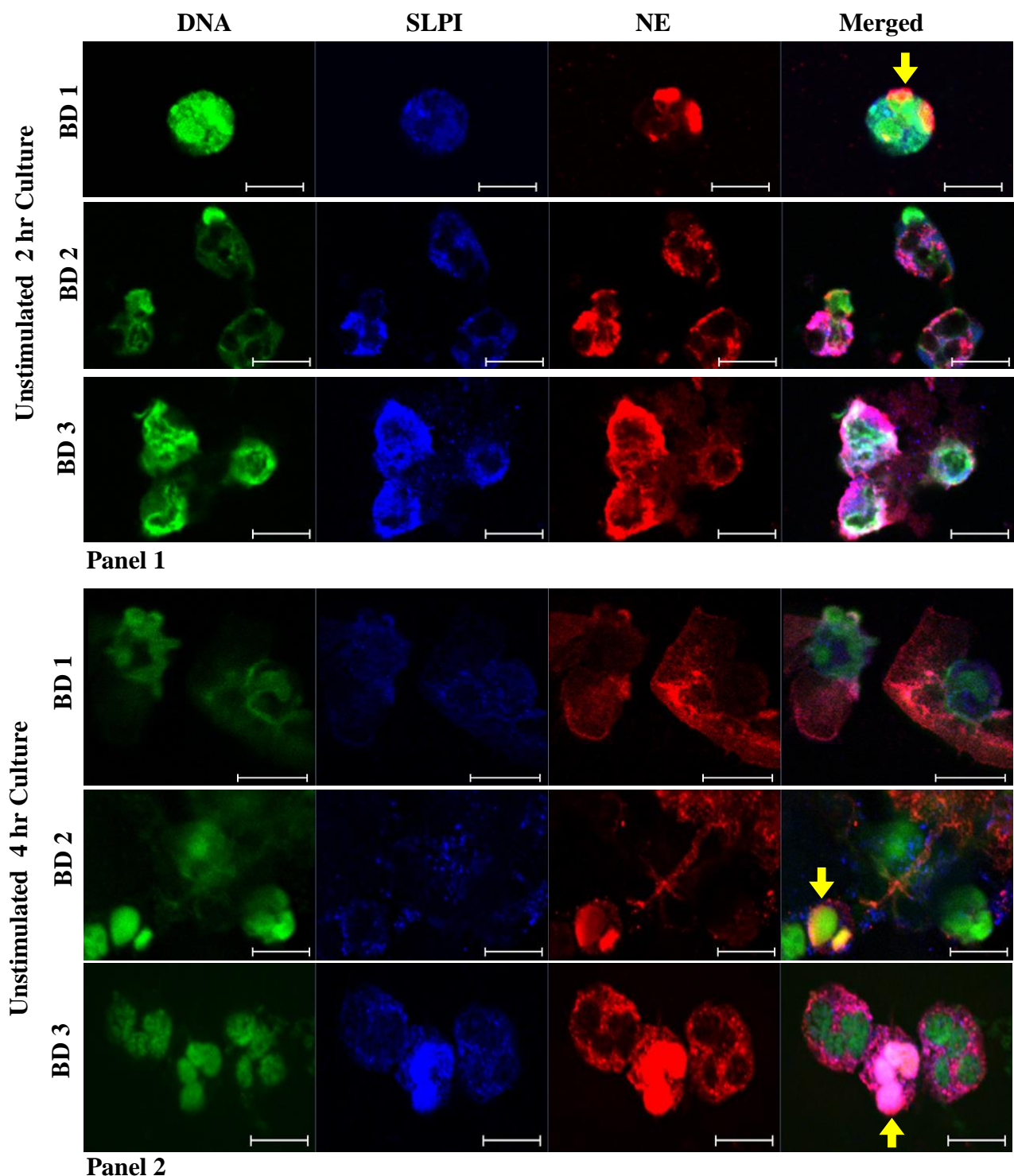


Fig 5.3.2.1.2.1.2. Non-Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in an Unstimulated Culture. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were incubated for a total of 2 hrs (Panel 1) and 4 hrs (Panel 2) in culture media. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). Yellow arrows indicate definitive stages of NETosis. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

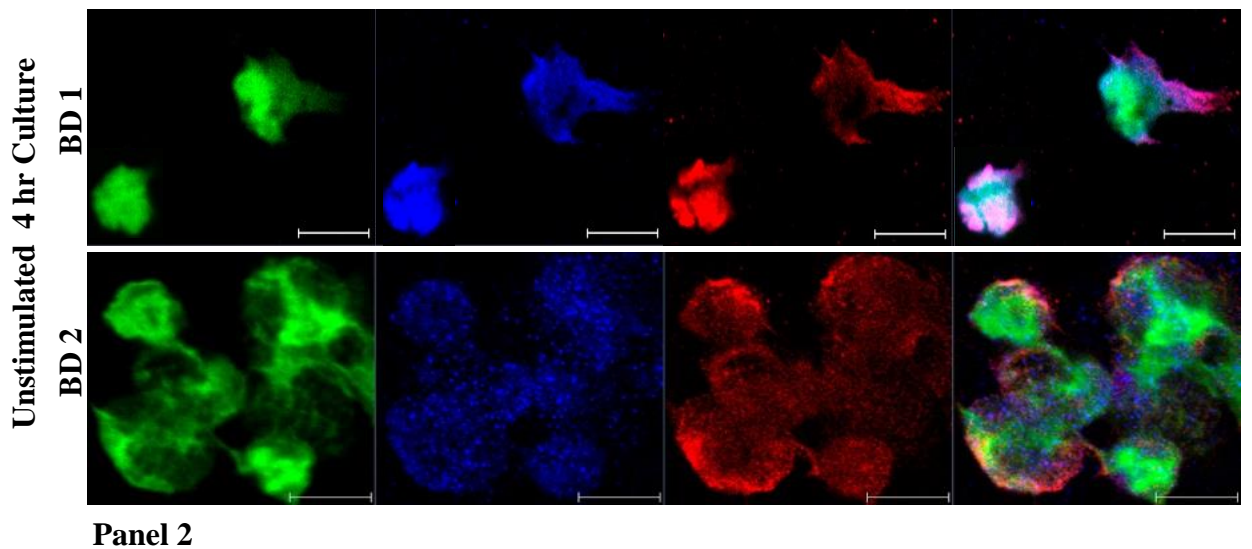
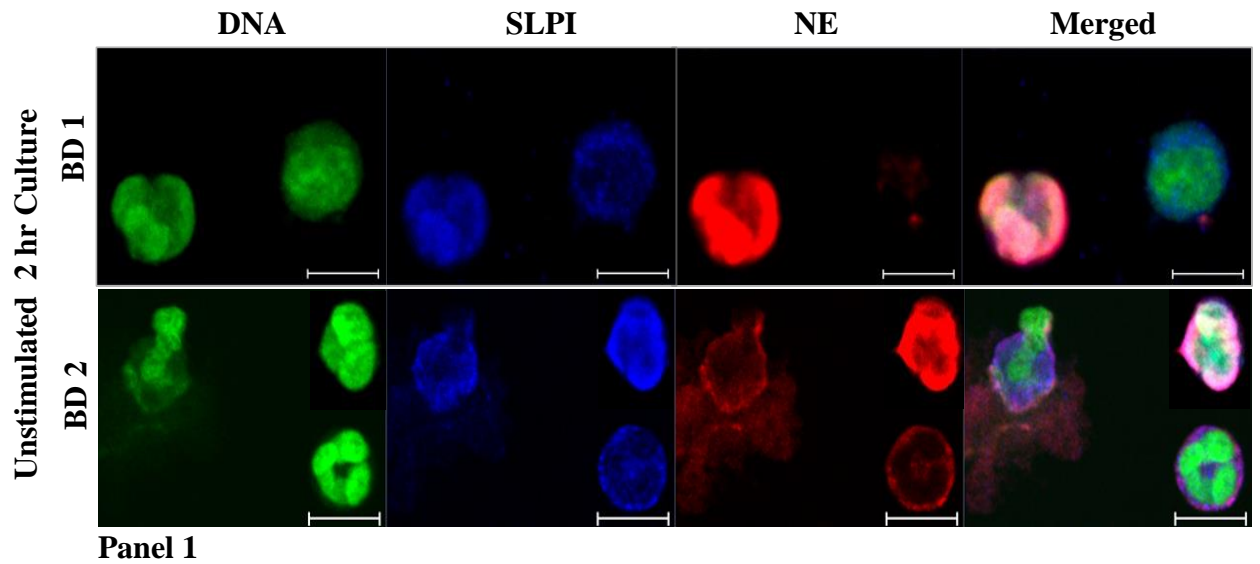
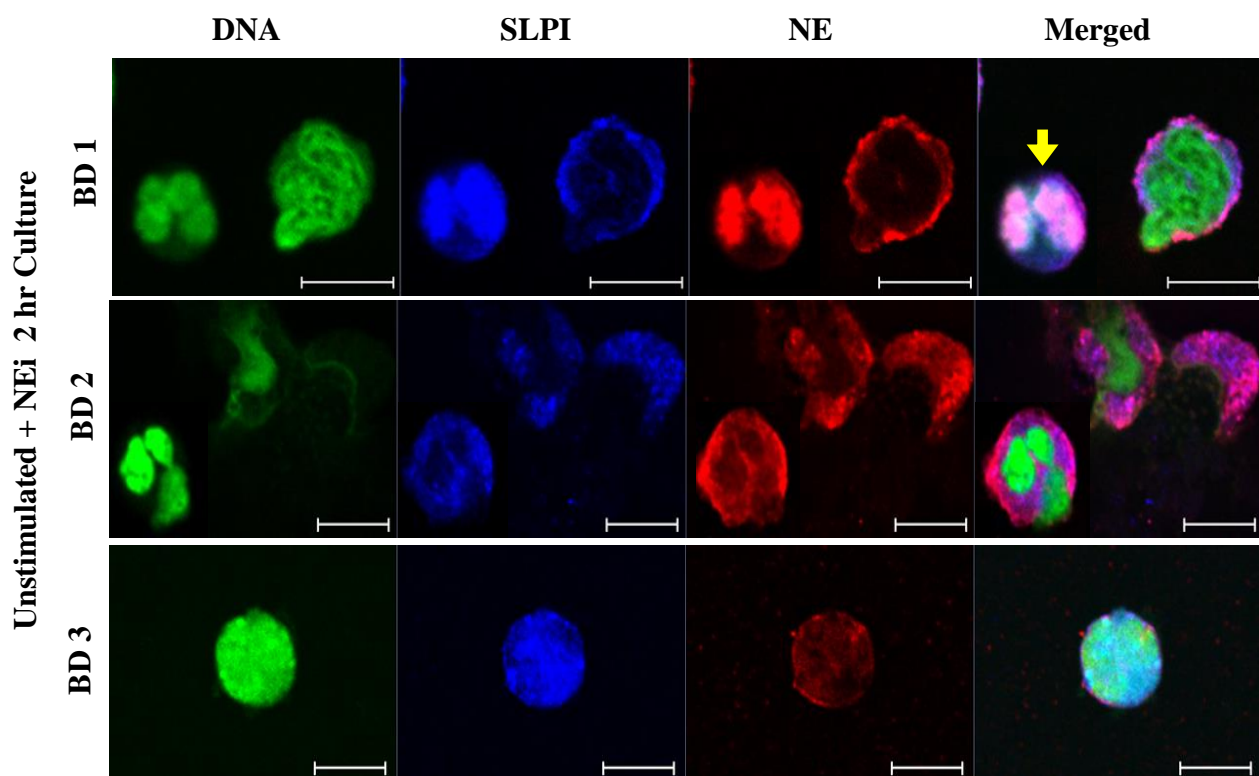
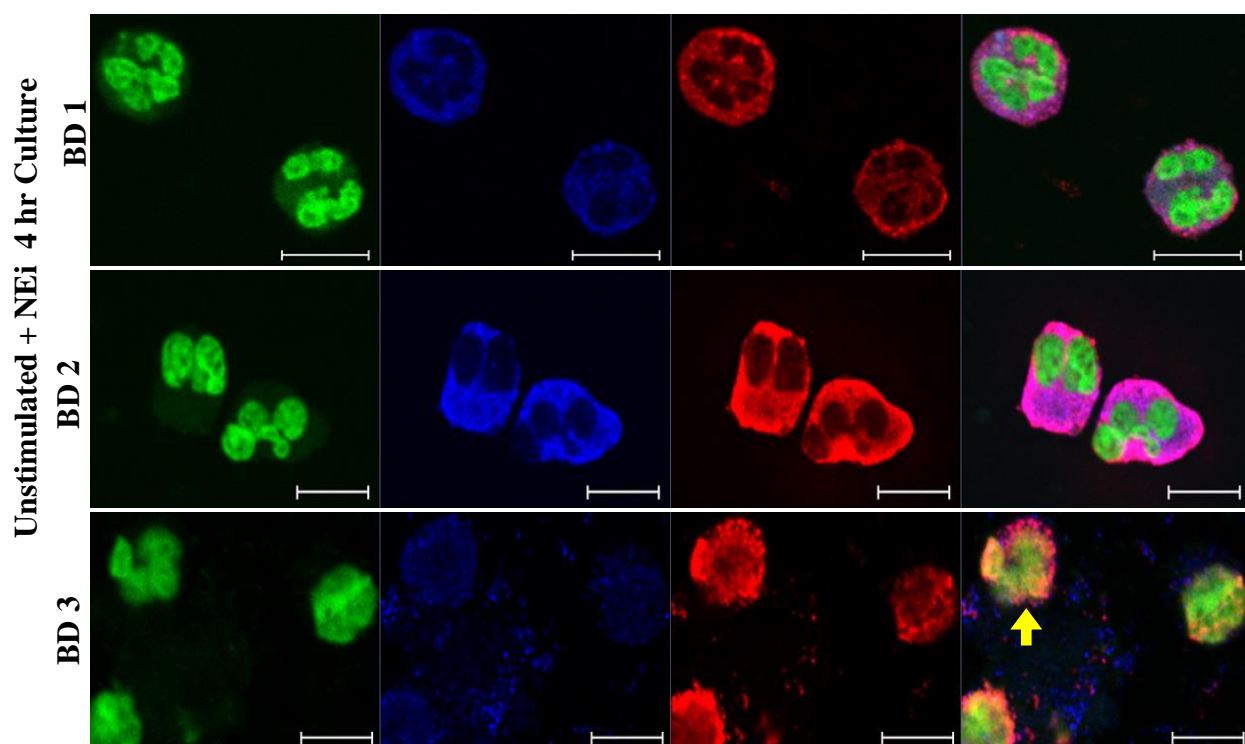


Fig 5.3.2.1.2.1.3. Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in an Unstimulated Culture. Neutrophils incubated for a total of 2 hrs (Panel 1) and 4 hrs (Panel 2) in culture media. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.



Panel 3



Panel 4

Fig 5.3.2.1.2.2.2. Non-Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in an Unstimulated Culture with NE Inhibitor. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were incubated for a total of 2 hrs (Panel 3) and 4 hrs (Panel 4) in culture media with NEi. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). Yellow arrows indicate definitive stages of NETosis. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

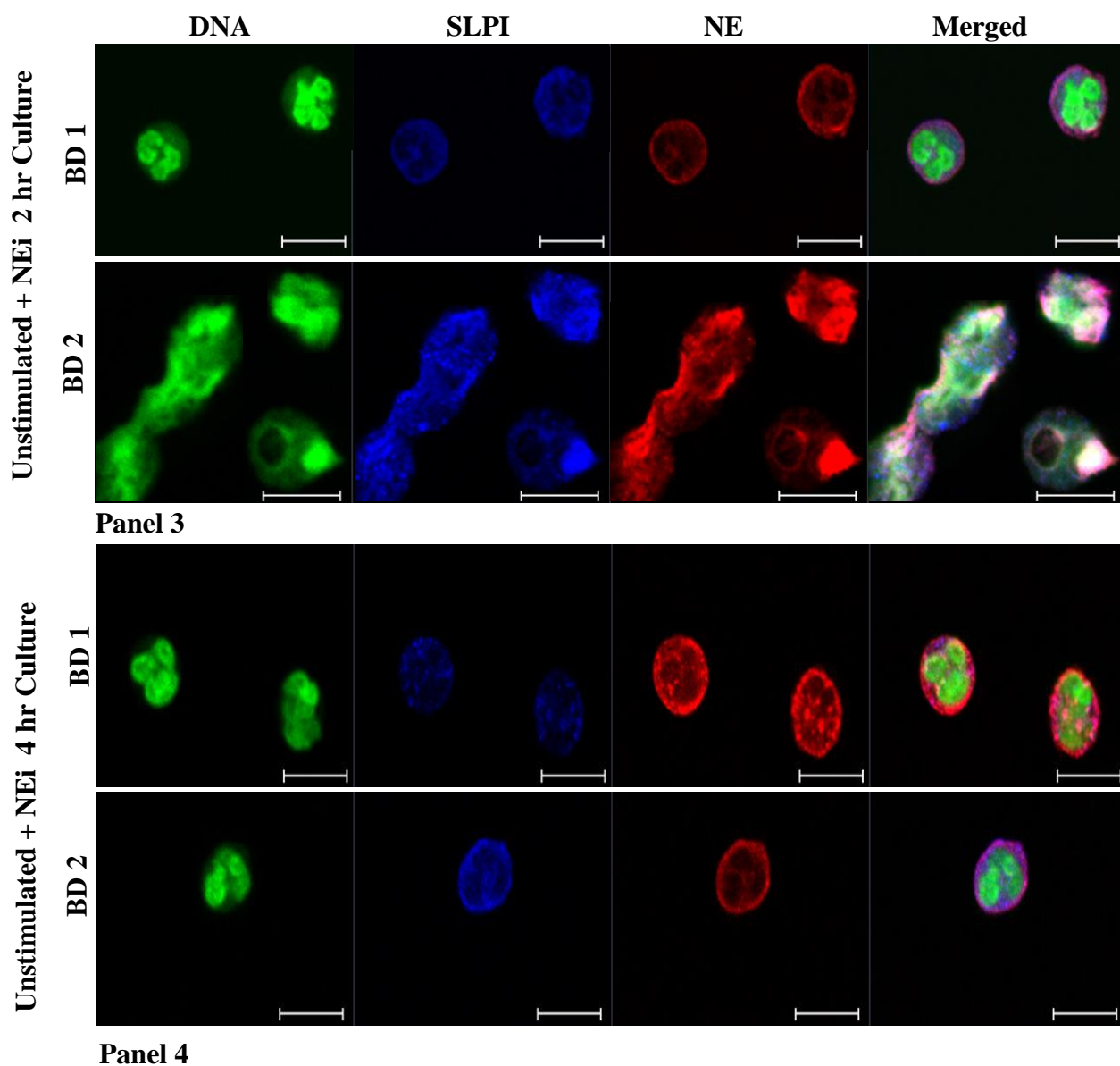
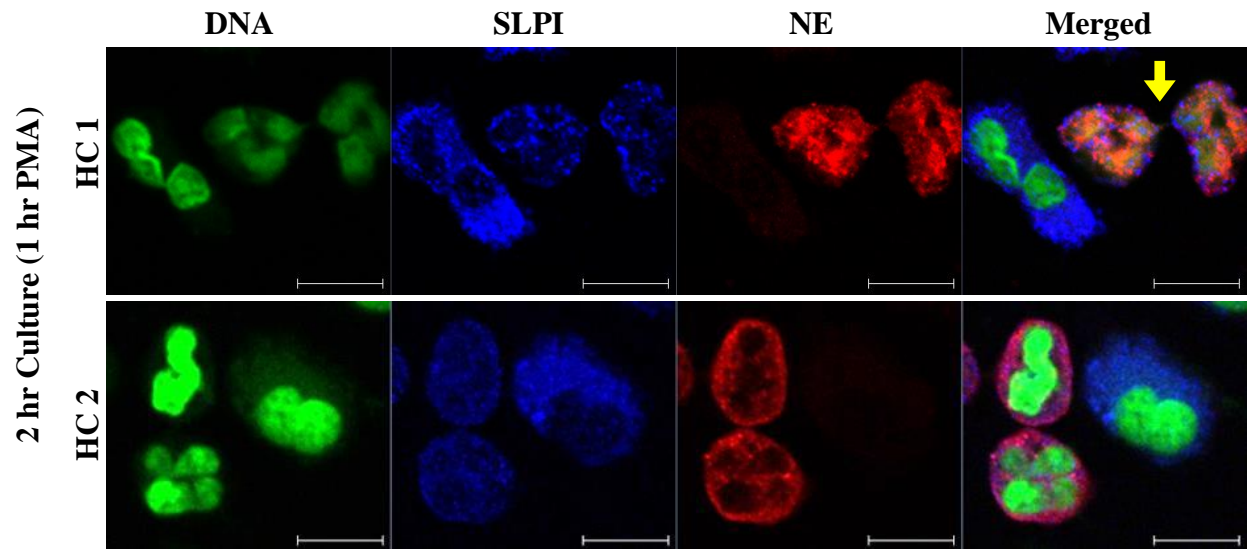
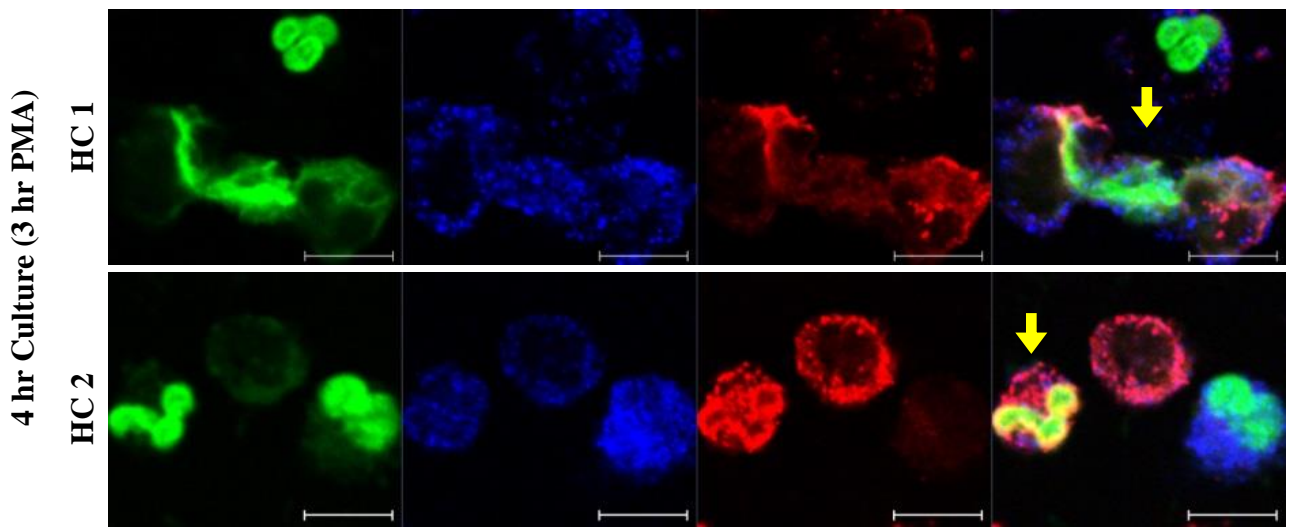


Fig 5.3.2.1.2.2.3. Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in an Unstimulated Culture with NE Inhibitor. Neutrophils were incubated for a total of 2 hrs (Panel 1) and 4 hrs (Panel 2) with NEi in culture media. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μm .

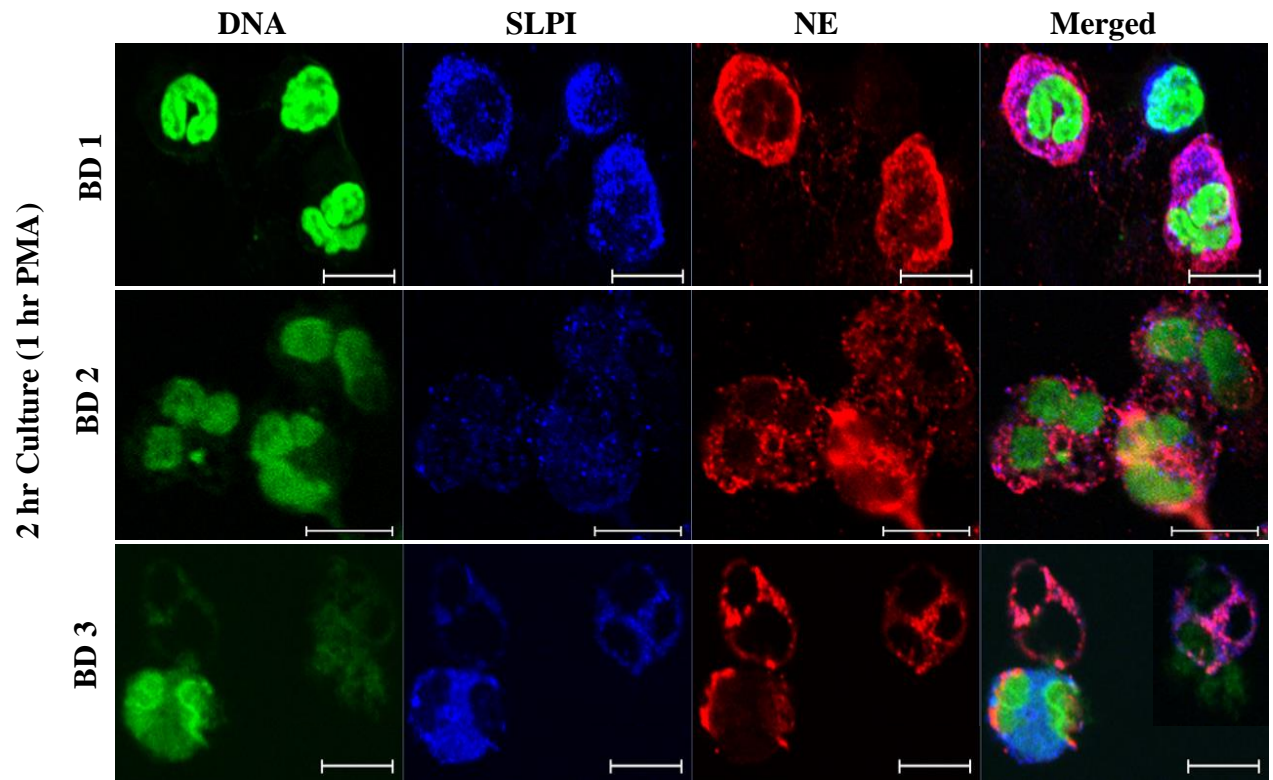


Panel 5

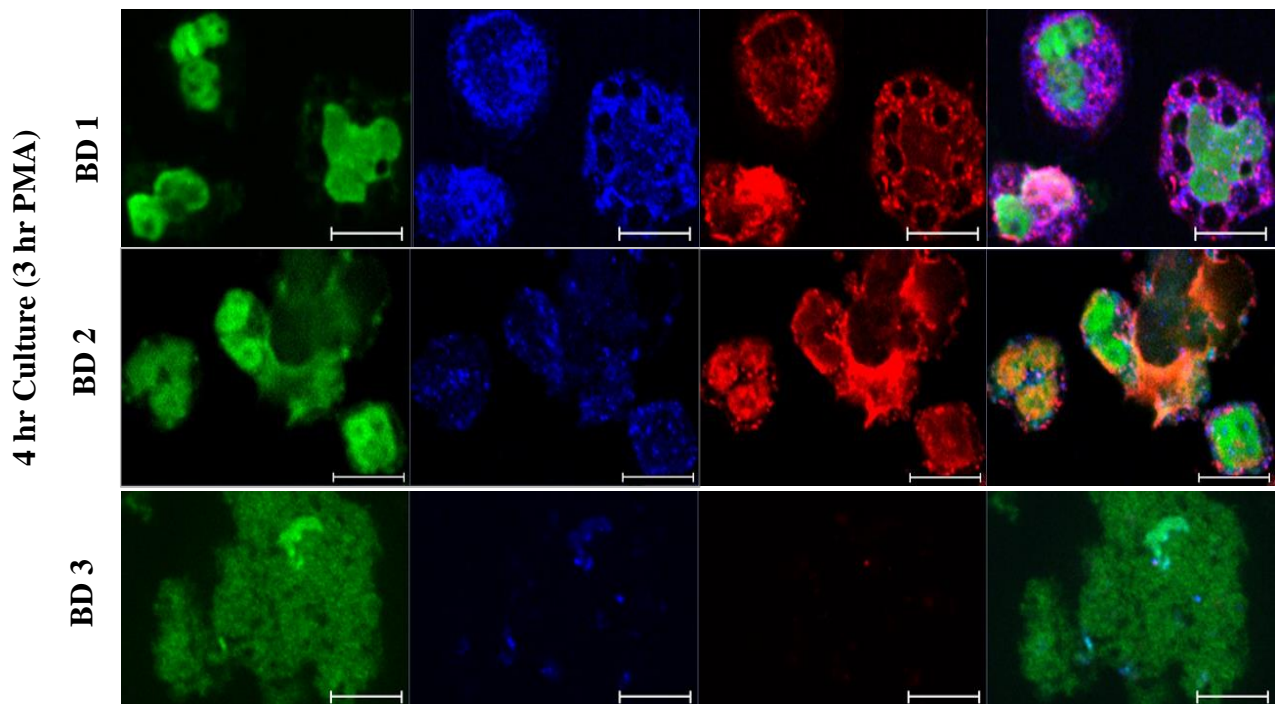


Panel 6

Fig 5.3.2.1.2.3.1. HC Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs (Panel 5) and 4 hrs (Panel 6) in culture media without NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). Yellow arrows indicate various stages of NETosis. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

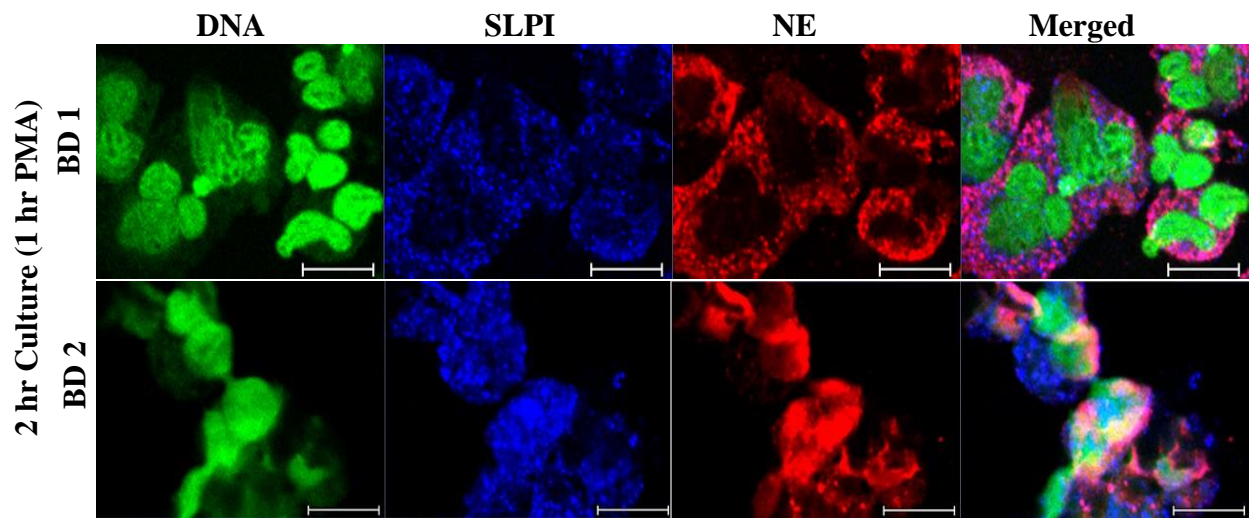


Panel 5

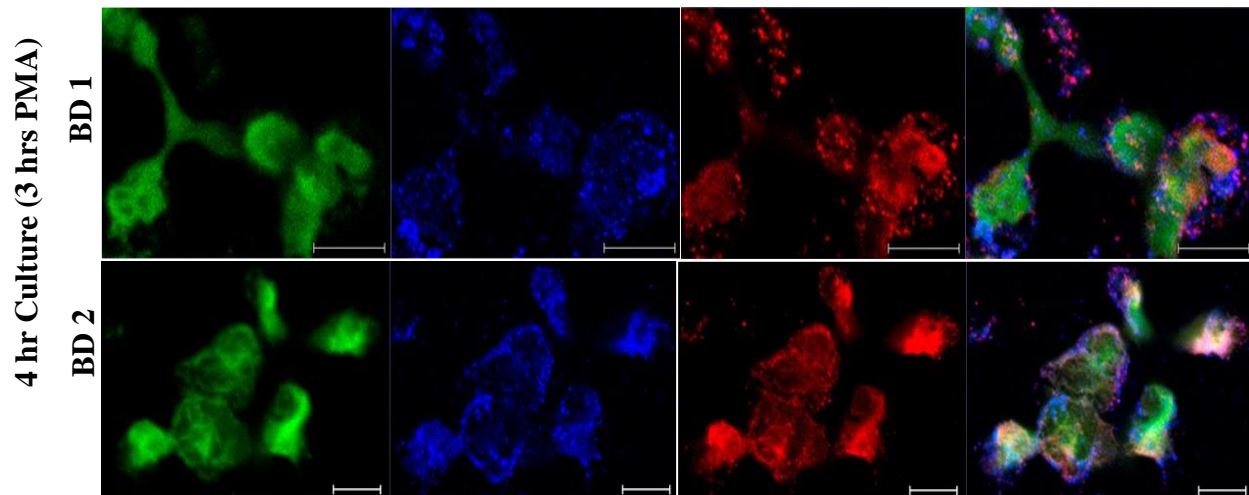


Panel 6

Fig. 5.3.2.1.2.3.2. Non-Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs (Panel 5) and 4 hrs (Panel 6) in culture media without NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.



Panel 5



Panel 6

Fig 5.3.2.1.2.3.3. Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture. Neutrophils were incubated for a total of 2 hrs (Panel 5) and 4 hrs (Panel 6) in culture media without NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

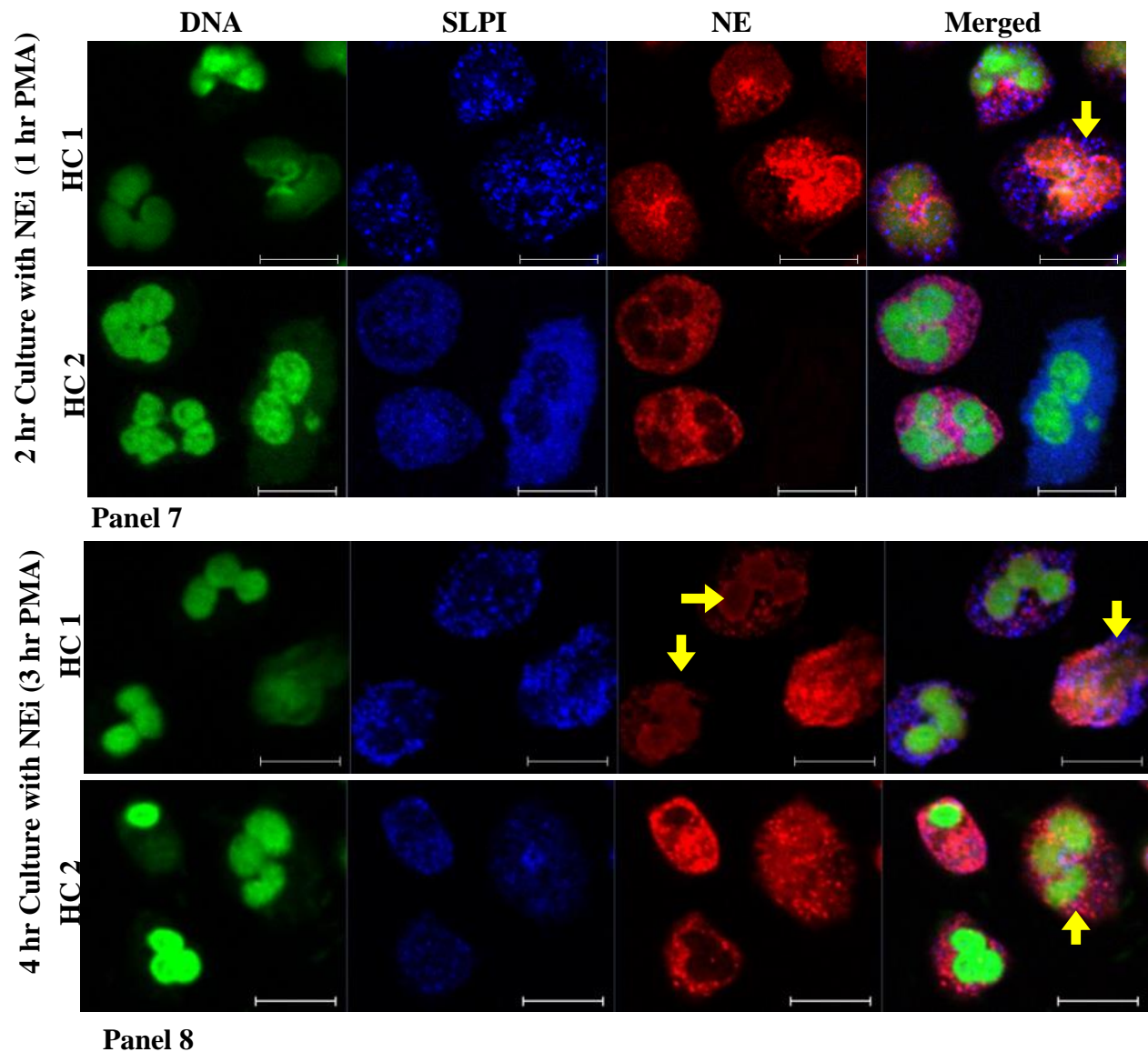
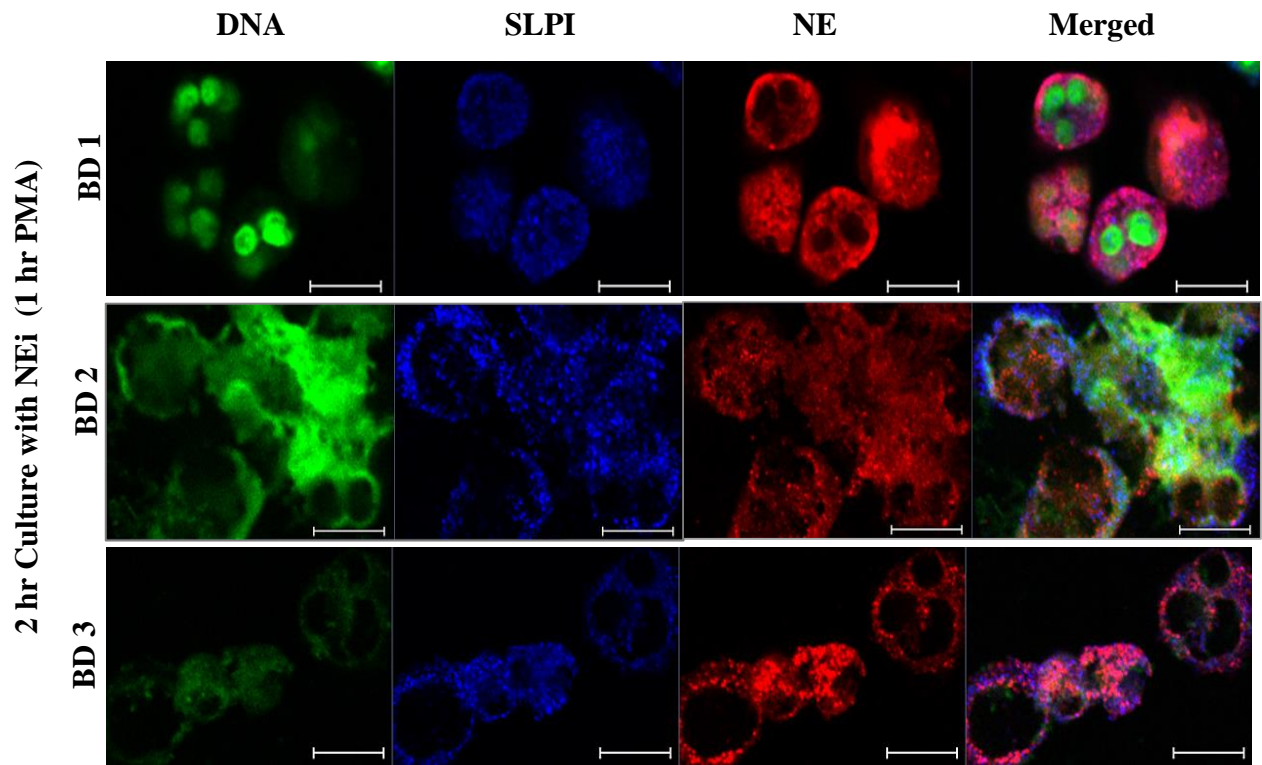
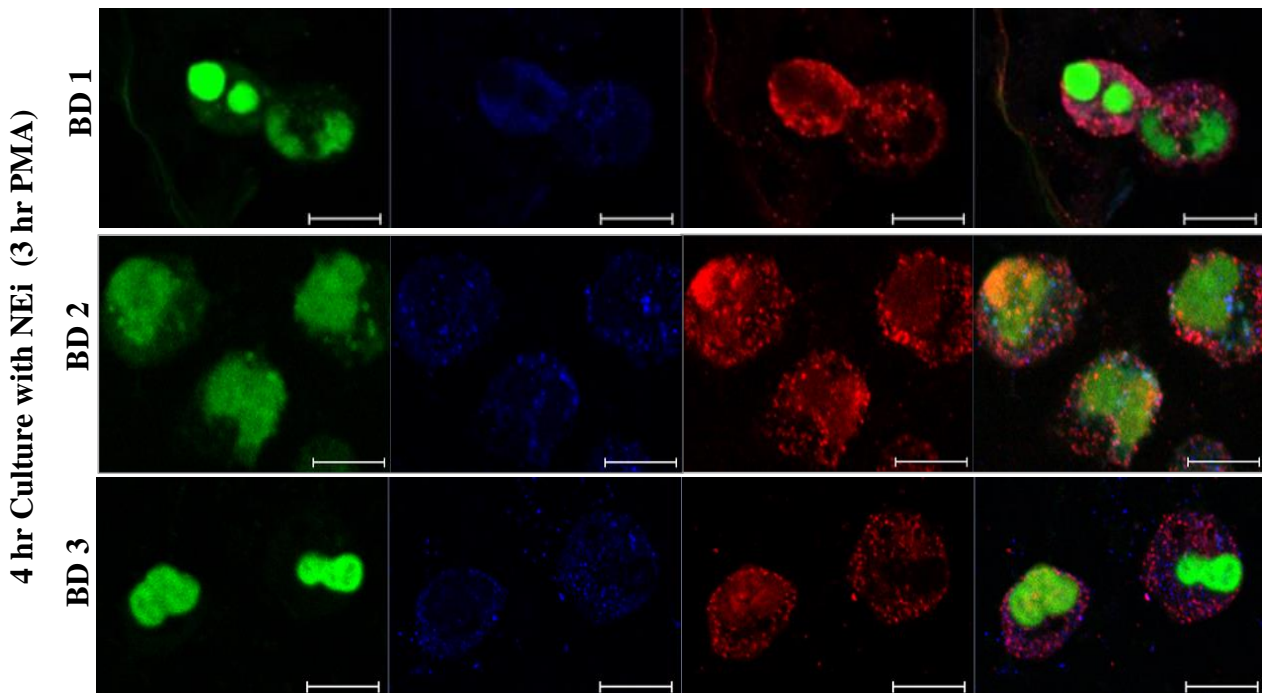


Fig 5.3.2.1.2.4.1. HC Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture with NE Inhibitor. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs (Panel 7) and 4 hrs (Panel 8) in culture media with NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The neutrophils were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). Yellow arrows indicate various stages of NETosis. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

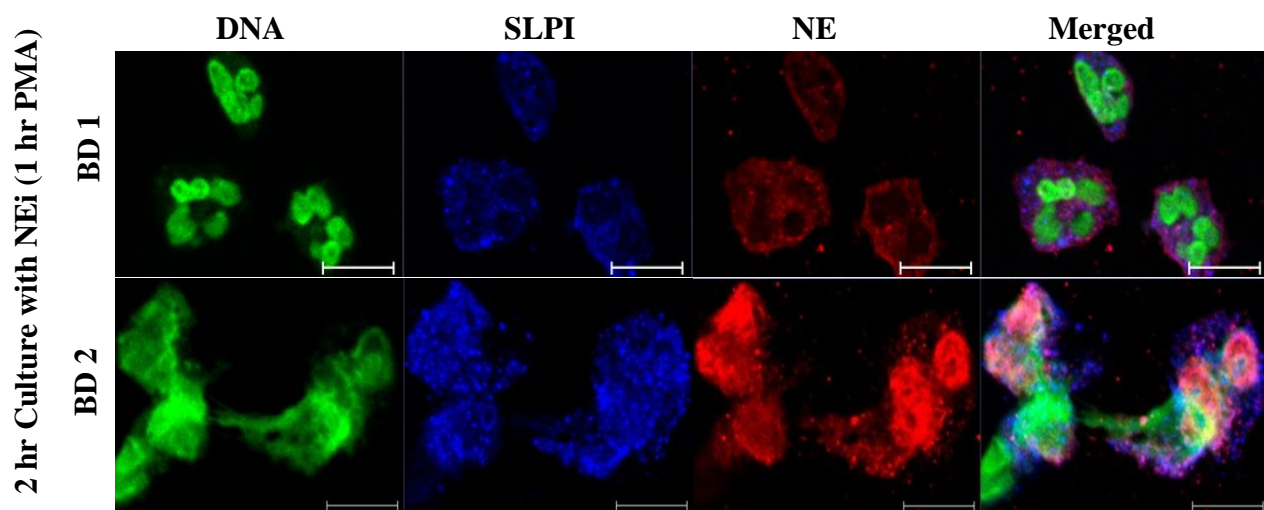


Panel 7

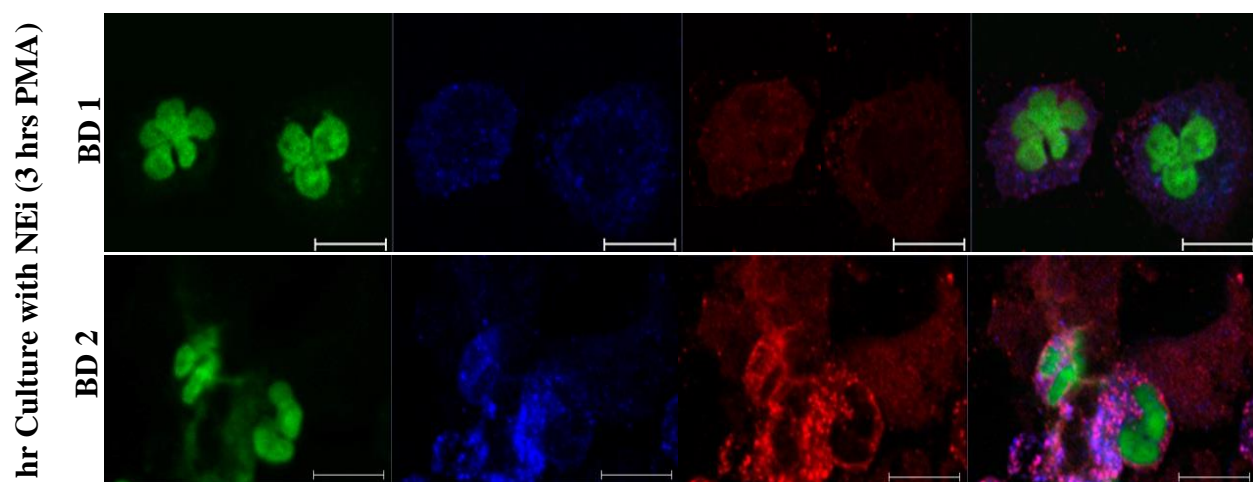


Panel 8

Fig 5.3.2.1.2.4.2. Non-Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture with NE Inhibitor. Neutrophils were incubated for a total of 2 hrs (Panel 7) and 4 hrs (Panel 8) in culture media with NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.



Panel 7



Panel 8

Fig 5.3.2.1.2.4.3. Relapsed BD Patients' Neutrophils *In Vitro*: Investigating the Occurrence of NETosis in a PMA-Stimulated Culture with NEi. Neutrophils were incubated for 2 hrs (Panel 7) and 4 hrs (Panel 8) in culture media with NEi. 100 nM of PMA was added after the first hour of incubation. Cells were then fixed in 4% PFA. The cells were stained for NE (red) and SLPI (blue) and DNA (DAPI) (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

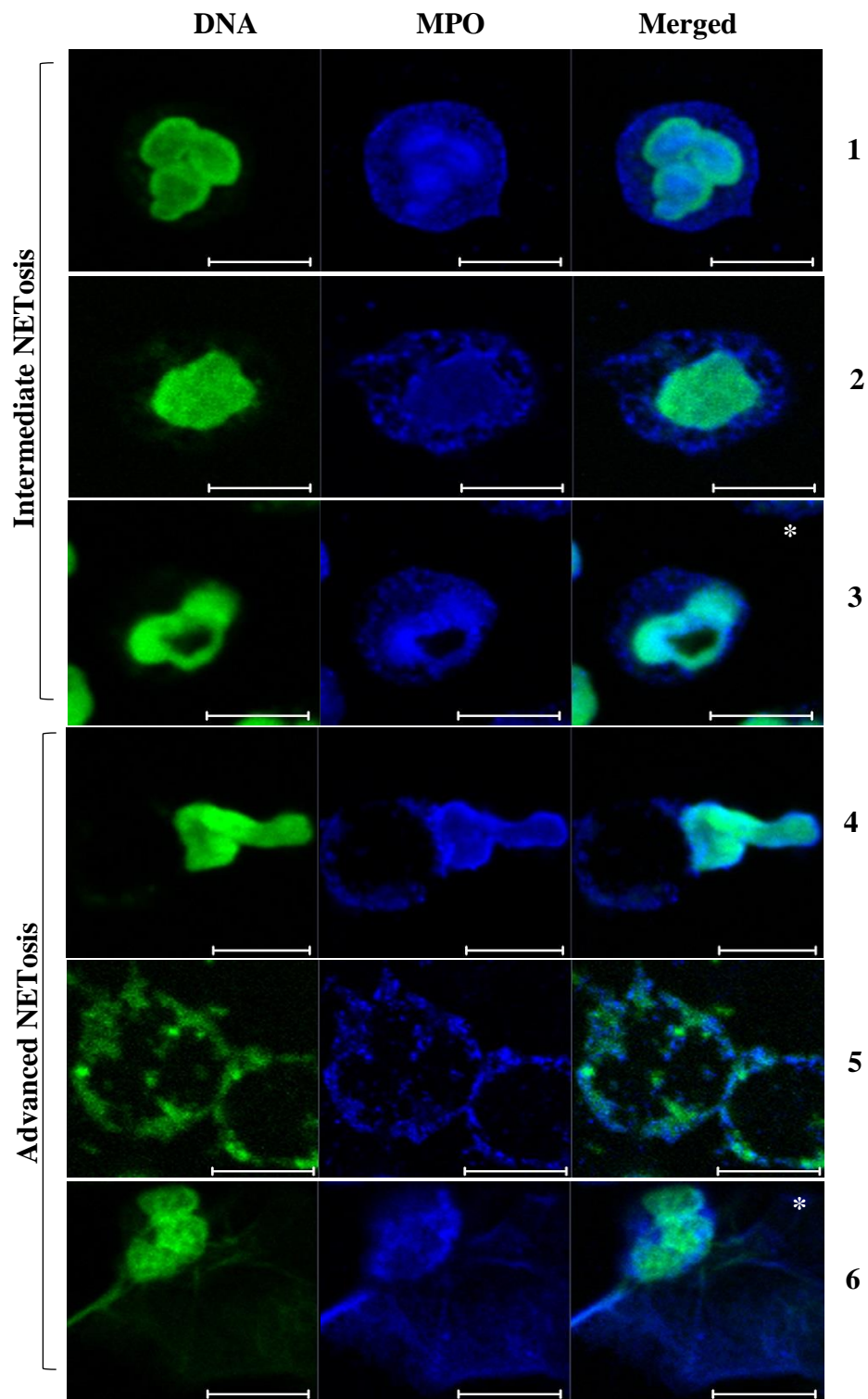


Fig 5.3.2.1.3.1. Positive Control: Neutrophils Undergoing NETosis in a PMA-Stimulated Culture. Demonstrated in the panels above are the typical morphologies of NETosing HC and BD neutrophils when they were induced with PMA. The main MPO activity occurred in the intermediate stage of NETosis after NE had translocated into the nucleus, therefore the first image shows this integration of MPO (blue) into the nucleus. The second and third panels show progressed intermediate stages in which MPO granules in the cytoplasm become more concentrated and pronounced, decondensation of DNA (green) takes place, and the cell continues to swell and vacuoles may develop both in the cytoplasm and nucleus. The final panel shows the advanced stage of NETosis in which cells erupts and casts out NETs. The asterisk marked panels signify the commonly observed progression of NETosis from decondensed nuclei to advanced NETosis. Confocal 710, X40 oil magnification. Bar indicated 10 μ m.

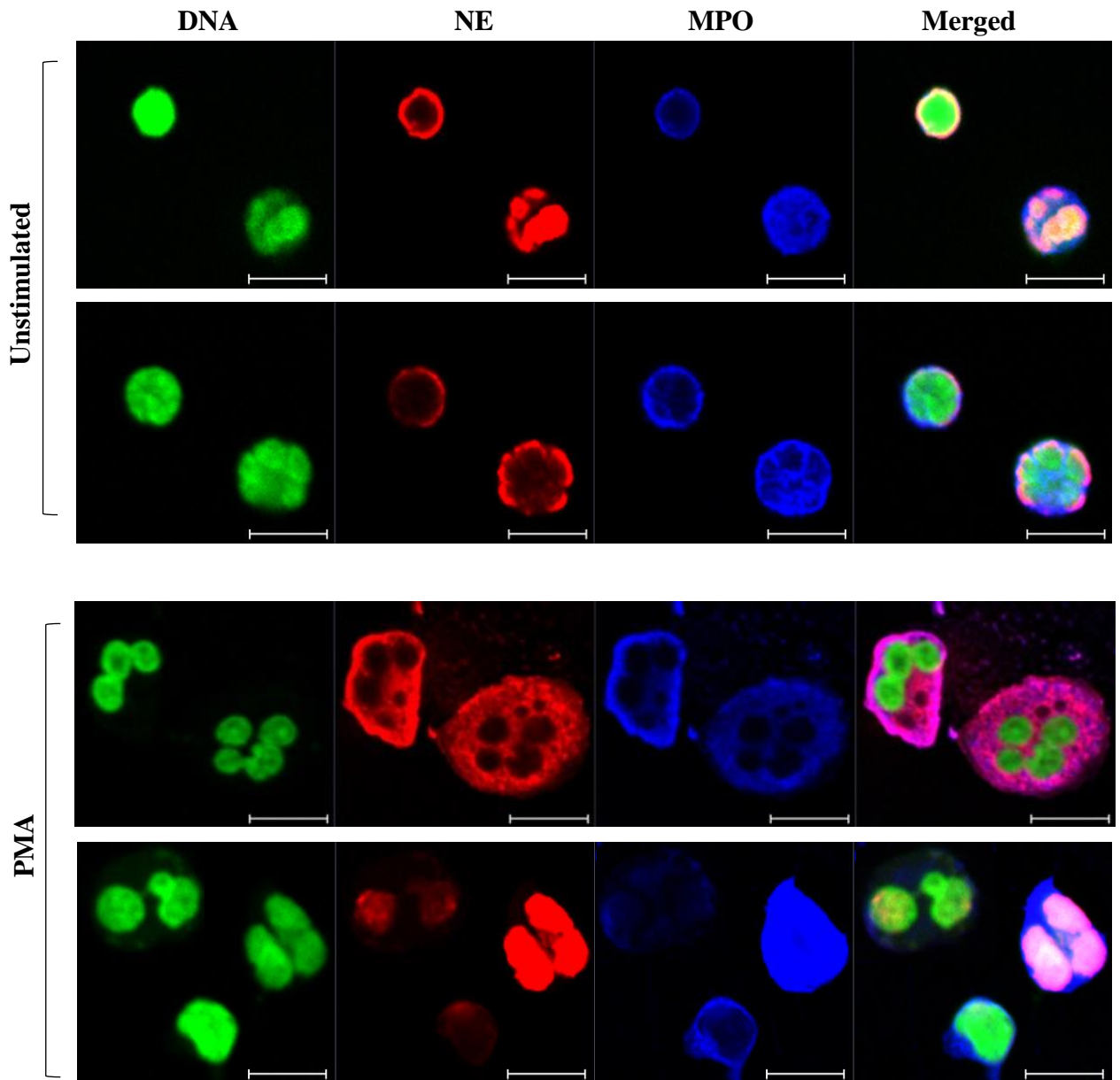


Fig 5.3.2.1.3.2. Unstimulated and PMA-Stimulated HC Neutrophils *In Vitro*: Observation of MPO. Neutrophils were cultured in 24-well plates on non-coated glass coverslips. The cells were all incubated for a total of 2 hrs and 4 hrs in culture media before being fixed in 4% PFA. The cells were stained for NE (red), MPO (blue) and DNA (green). Co-localisation of all three targets produced pink regions. X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

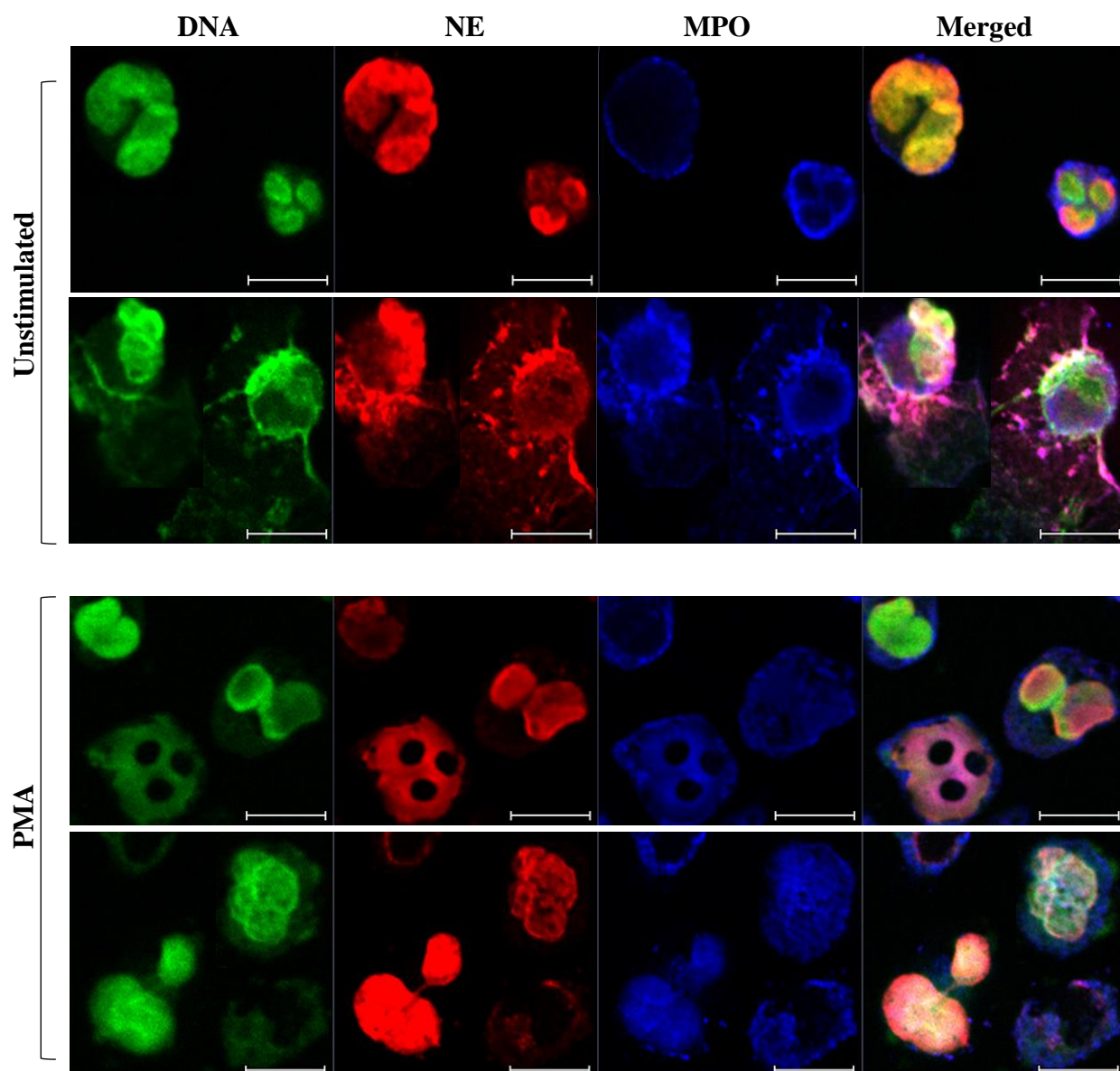


Fig. 5.3.2.1.3.3. Unstimulated and PMA-Stimulated Non-Relapsed BD Neutrophils *In Vitro*: Observation of MPO. Neutrophils were incubated for a total of 2 and 4 hrs in culture media before being fixed in 4% PFA. The cells were stained for NE (red), MPO (blue) and DNA (green). Co-localisation of all three targets produced pink regions. X40 oil magnification, Confocal 710. Bar indicates 10 μm.

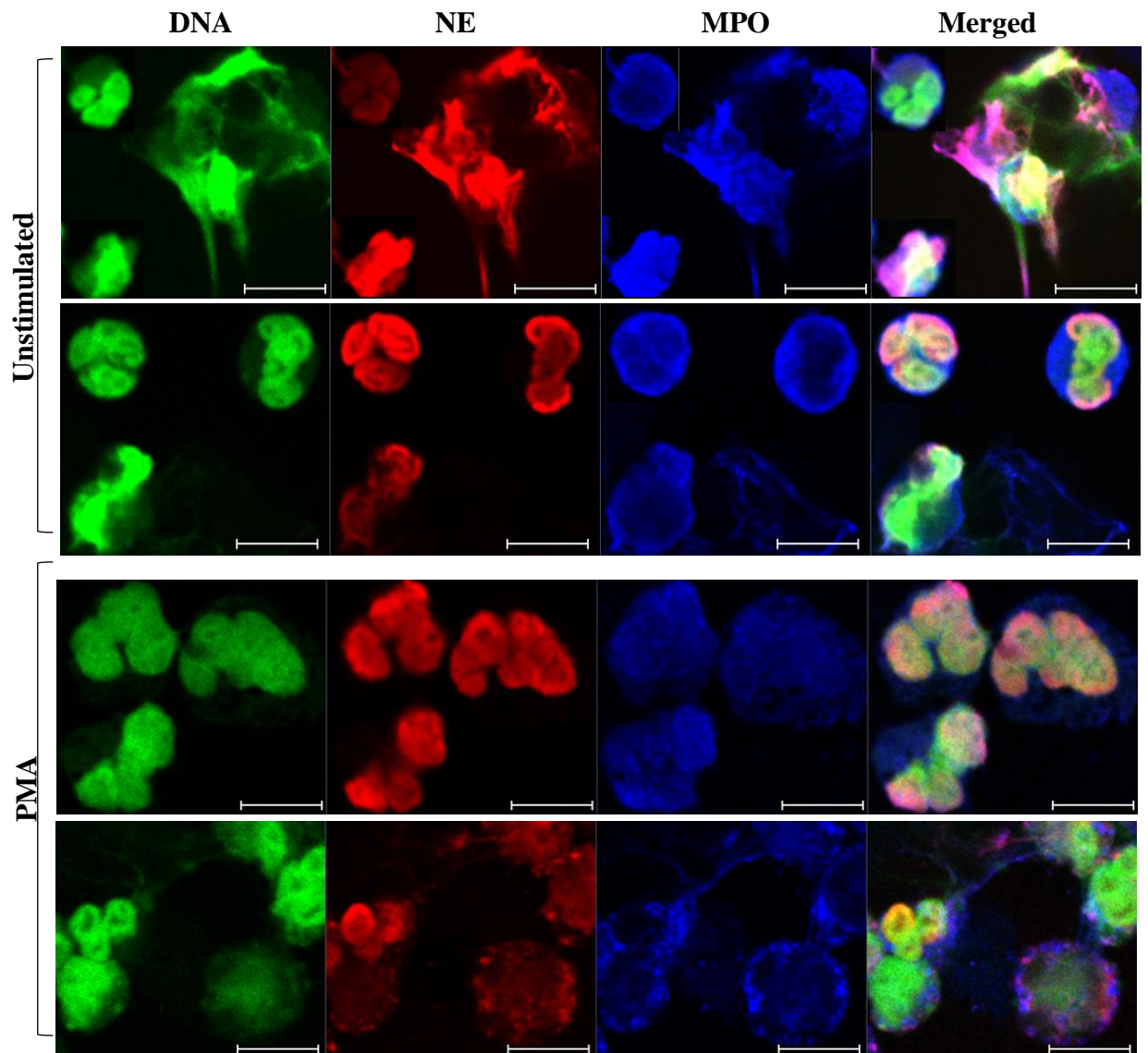


Fig 5.3.2.1.3.4. Unstimulated and PMA-Stimulated Relapsed BD Neutrophils *In Vitro*: Observation of MPO. Neutrophils were incubated for a total of 2 and 4 hrs in culture media before being fixed in 4% PFA. The cells were stained for NE (red), MPO (blue) and DNA (green). Co-localisation of all three targets produced pink regions. X40 oil magnification, Confocal 710. Bar indicates 10 μm.

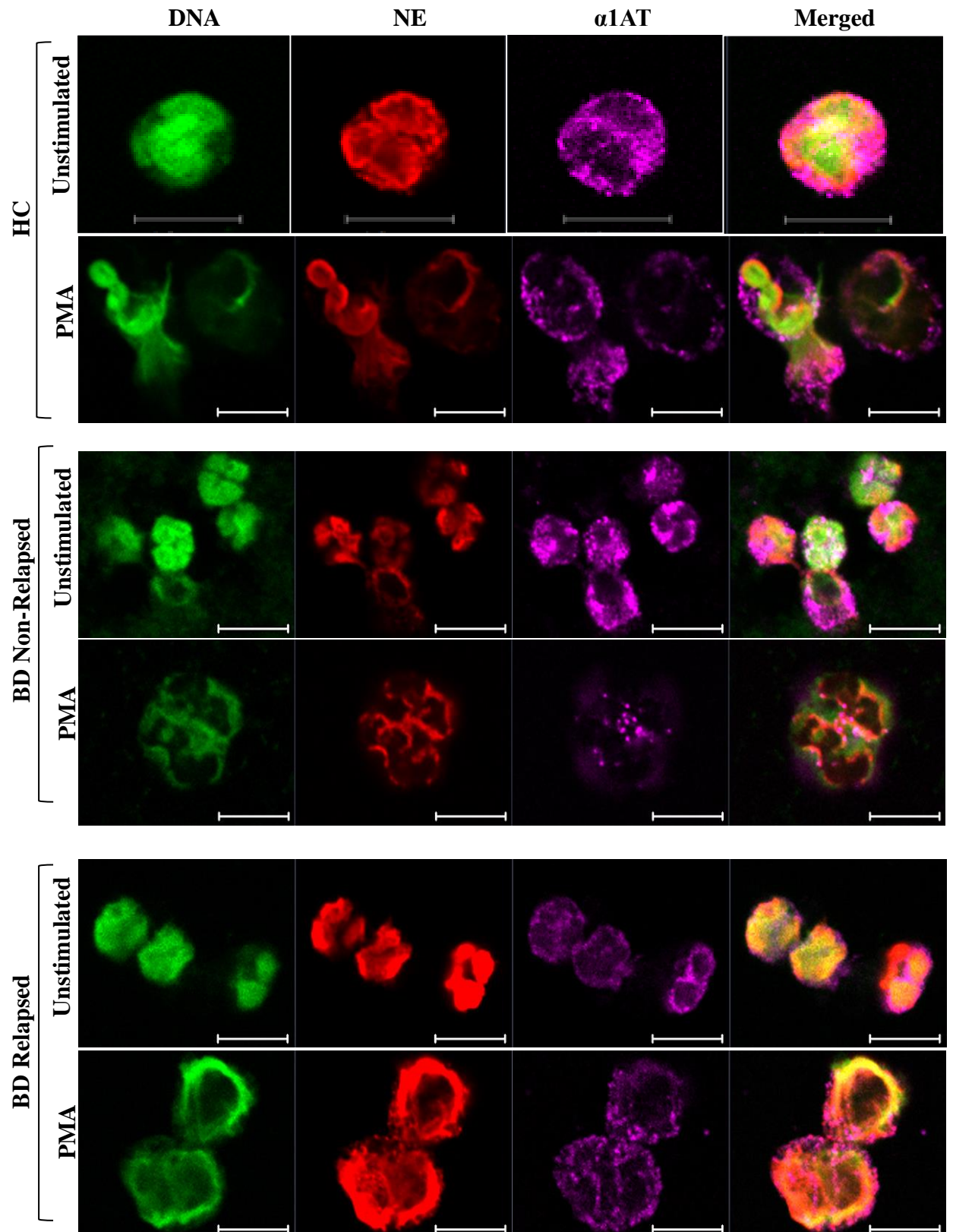


Fig. 5.3.2.1.4.1. Alpha-1Antitrypsin during NETosis *In Vitro*. HC, BD non-relapsed and relapsed patients' neutrophils were incubated with and without PMA then PFA-fixed and stained for NE (red), α 1AT (purple), DNA (green). X40 oil magnification, Confocal 710. Bar indicates 10 μ m.

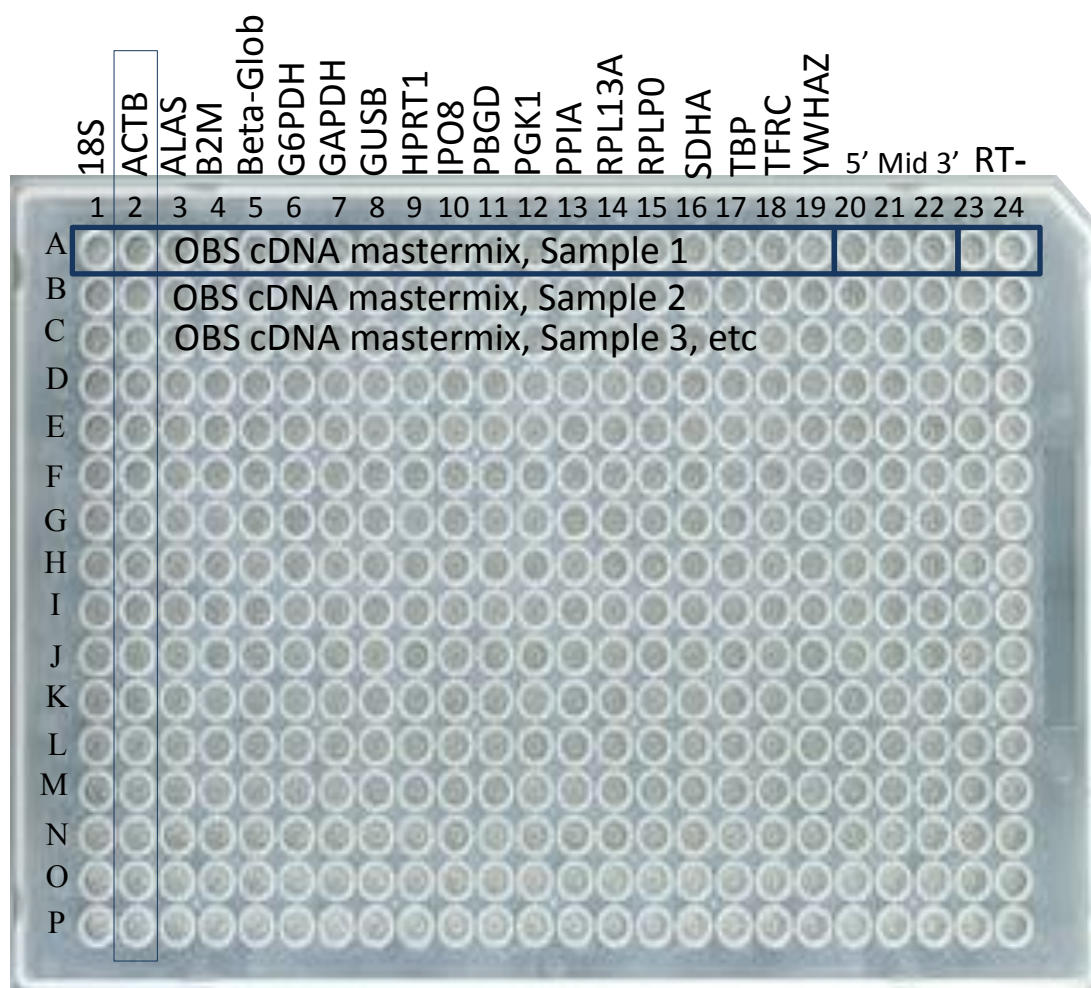


Fig 6.2.1.3. The RealTime Ready Human Reference Gene 384-well plate. 10 μ l of each cDNA OBS sample mastermix was added to each row from wells 1 – 22. Each column contained a different pre-absorbed specific primers for each reference gene target. Untranscribed RNA from the same OBS sample, previously set aside after the extraction, was added to wells 23-24. This consisted of 1 μ l of the RNA, 4 μ l of RNase free H₂O, and 5 μ l of the 2X LightCycler[®] 480 Probes Master reaction mix.